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(54) Title: THE USE OF HUMAN FGH-8 POLYPEPTIDES AS NEUROTROPHIC AGENTS

(57) Abstract: The present invention relates to pharmaceutical compositions and methods of treating neurological disorders. Compositions of the present invention comprise at least one hFGF-8 polypeptide, or at least one isolated nucleic acid, or its complement, encoding at least one hFGF-8 polypeptide. The present invention also includes hFGF-8 antibodies and chimerics, hFGF-8 expressing vectors, host cells and transgenics, as well as methods of making and using thereof.

THE USE OF HUMAN FGF-8 POLYPEPTIDES AS NEUROTROPHIC AGENTS

This invention relates to recombinant DNA technology. In particular the invention pertains to pharmaceutical and other uses of human FGF-8 genes and their encoded proteins. The compounds and compositions of the present invention can be used alone or in conjunction with other neurotrophic, neuroprotective, thrombolytic, and/or anti-thrombotic agents, in methods for treating or preventing neuronal damage caused by disease or trauma.

Stroke is the third leading cause of death and disability in the United States. With an excess of 500,000 new occurrences each year, strokes are responsible for about 300,000 deaths annually in the U.S. alone. Strokes are also a leading cause of hospital admissions and long-term disabilities. Accordingly, the socioeconomic impact of stroke and its attendant burden on society is immense.

" Stroke" is defined by the World Health Organization as a rapidly developing clinical sign of focal or global disturbance of cerebral function with symptoms lasting at least 24 hours. More than 85% of strokes are due to a thrombo-embolic occlusion of a cerebral artery, resulting in death of brain tissue. With complete occlusion, arrest of

cerebral circulation causes cessation of neuronal electrical activity within seconds. Within a few minutes after the deterioration of the energy state and ion homeostasis, depletion of high energy phosphates, membrane ion pump failure, efflux of cellular potassium, influx of sodium chloride and water, and membrane depolarization occur. If the occlusion persists for more than five to ten minutes, irreversible damage results. Although severe ischemia can be lethal, generally, ischemia is not total after a thrombotic occlusion of a cerebral vessel. Even with incomplete ischemia, however, the outcome is difficult to evaluate and depends largely on residual perfusion and the availability of oxygen.

When the ischemic event is moderate it generally results in a region of collaterally perfused tissue having paralyzed neuronal function surrounding a core of severe ischemia where the infarct developed. The zone of damaged brain tissue, called a " penumbra" , consists of brain tissue in a state between life and death. Oftentimes, the loss of neuronal function can be reversed with restoration of adequate perfusion.

Cerebral infarction commonly results in deficits of motor, sensory, visual, or cognitive function. Recovery involves dendritic and axonal sprouting and new synapse formation in the damaged tissue or other hemisphere. The eventual extent of neurologic recovery depends on the patient's age and general state of health as well as on the site and size of the infarction. Impaired consciousness, mental deterioration, or aphasia, suggest a poor prognosis. About 50% of patients with moderate or severe hemiplegia, and most of those with lesser deficits, recover functionally and are ultimately able to care for their basic needs, have

a clear sensorium, and can walk adequately, although use of an affected limb may be limited. Although some patients continue to improve slowly, any deficit remaining after 6 months is likely to be permanent. Furthermore, recurrence of cerebral infarction is relatively common, and each recurrence is likely to add to the neurologic disability.

In addition to stroke, other neurological diseases are also associated with the death of or injury to neuronal cells. For example, Parkinson's disease results from the loss of dopaminergic neurons in the substantia nigra. Although the molecular mechanism of neurodegeneration in Alzheimer's disease is yet to be established, inflammation and deposition of beta-amyloid protein and other such agents may compromise neuronal function or survival. In patients suffering from brain trauma or spinal cord injuries, extensive neuronal cell death is also observed. Nitric oxide (NO) and oxidative stress are thought to play important roles in the pathogenesis of many neurodegenerative disorders including neuronal dysfunction after stroke or head trauma, retinal degeneration, Alzheimer's disease and Parkinson's disease. NO also plays a role in neuropathic pain, migraine, psychoses, angiogenesis, and vasculogenesis.

Although, there are presently no satisfactory treatments for these diseases the use of compounds capable of inhibiting neuronal cell death, promoting nerve regeneration, stimulating neurite outgrowth, and inhibiting NO mediated toxicity are promising approaches to treatment of such disorders.

In individuals suffering from a neurological disease, an induction of neurite outgrowth may protect neurons from further degeneration, and accelerate the regeneration of

nerve cells. Neurite outgrowth may be stimulated in vitro by various growth factors. For example, Glial Cell Line-Derived Neurotrophic Factor (GDNF) demonstrates neurotrophic activity both, *in vivo* and *in vitro*, and is currently being investigated for the treatment of Parkinson's disease. Insulin and insulin-like growth factors have been shown to stimulate growth of neurites in rat pheochromocytoma PC12 cells and in cultured sympathetic and sensory neurons (Recio-Pinto et al., J. Neurosci., 6:1211-1219 (1986)). Similarly, fibroblast growth factor-2 (bFGF) stimulates neural proliferation and growth (D. Gospodarowicz, *et al.*, Cell Differ., 19:1 (1986); M. A. Walter et al:, Lymphokine Cytokine Res., 12:135 (1993)), enhances neuronal sprouting *in vitro* , and stimulates functional recovery in neuronal recuperation models (Kawamata, *et al.*, J. Cereb. Blood Flow Metab., 16:542-547, (1996); Kawamata, *et al.*, Proc. Natl. Acad. Sci. USA, 94:8179:8184, (1997a)). Likewise, FGF-8 has been shown to play a critical role in growth and polarity of the developing midbrain (Lee et al. 1997). FGF-8 has also been shown to have direct biological activities on neurons, including increasing GABA uptake and CREB phosphorylation in rat cortical cultures (Green et al. 1998).

Originally identified in the conditioned medium of a murine androgen-dependent mammary carcinoma cell line (SC-3) (Tanaka, *et al.*, Biochem. & Biophys. Res. Comm., 204(2):882-8, (1994)), FGF-8 is composed of 215 amino acids and displays a 30-40% homology to other members of the FGF family. The human FGF-8 gene has been mapped to chromosome 10q24 and is induced by androgen in breast cancer cells (Payson, *et al.* 1996). FGF-8 is also subject to a complex alternative splicing process that generates transcripts with coding potential for different FGF-8 isoforms (Blunt, *et*

al., J. of Biol. Chem., 272(6):3733-8, (1997); Tanaka, *et al.* 1994; MacArthur, *et al.*, Cell Growth & Differentiation., 6(7):817-25 (1995); MacArthur et al., Genomics, 35(1):253-7, (1996); MacArthur, *et al.*, Development, 121(11):3603-13, (1995); Crossley and Martin 1995). Although the mouse FGF-8 gene encodes a total of eight isoforms, the human gene encodes only 4 isoforms (FGF-8a, b, e, and f) due to a premature stop codon in exon 1B ORF (see Figure 1; Gemel, *et al.*, *Genomics*, 35(1), 253-7, (1996)).

The human FGF-8a and FGF-8b are 100% identical to their mouse counterpart while the FGF-8e and FGF-8f are 98% identical to their mouse counterpart.

Presently, the pharmacological therapies for cerebral focal stroke and/or other neural inflictions are limited to symptomatic treatments that do not alter the development and/or fate of the damaged tissue. The present invention provides compounds useful for treating, inhibiting, and/or preventing the process of neuro-degeneration caused by disease or trauma.

The present invention provides compounds and pharmaceutical compositions comprising human fibroblast growth factor-8 (hFGF-8) polypeptides, or nucleic acids encoding hFGF-8 polypeptides, which possess neuronal activity. The compounds and compositions can be used either alone or in conjunction with at least one other neurotrophic, neuroprotective, thrombolytic, and/or anti-thrombotic agent, such as nerve growth factor, in methods for treating or preventing neuronal damage caused by disease or trauma.

The compositions and methods of the present invention may be particularly useful for treating or preventing neuronal damage resulting from NO mediated toxicity.

The compositions and methods of the present invention may be particularly useful for enhancing recovery of neuronal function.

The present invention provides, in one aspect, isolated nucleic acid molecules comprising polynucleotides, or complements thereof, encoding specific hFGF-8 polypeptides, as well as fragments, or variants thereof, which can be used in methods for treating or preventing neuronal damage caused by disease or trauma. Nucleic acids encoding hFGF-8 full-length polypeptides, domains, fragments and/or variants thereof corresponding to at least 90-100% of the contiguous amino acids of at least one of SEQ ID NOS:4, 5, 6, and 7, are provided as non-limiting examples of the compounds and compositions of the present invention.

The present invention also provides methods of using said nucleic acids, vectors comprising 1-40 of said isolated hFGF-8 nucleic acid molecules, and/or host cells containing said nucleic acids and/or recombinant vectors, to produce hFGF-8 nucleic acids and/or polypeptides for use in treating or preventing neuronal damage caused by disease or trauma.

The present invention provides hFGF-8 polypeptides or fragments thereof which are highly potent in inducing enhanced growth of axonal and dendritic neuronal processes.

The present invention also provides methods of using an isolated hFGF-8 polypeptide, comprising at least one fragment, domain, or specified variant of at least 90-100% of the contiguous amino acids of at least one of SEQ ID NOS:4, 5, 6, and 7 to treat or prevent neuronal damage caused by disease or trauma.

The present invention also provides methods of using an isolated hFGF-8 polypeptide as described herein, wherein the polypeptide further comprises at least one substitution, insertion, or deletion corresponding to portions or residues of at least one of SEQ ID NOS:4, 5, 6, and 7 to treat or prevent neuronal damage caused by disease or trauma.

The present invention also provides a method of using an isolated hFGF-8 polypeptide, as described herein, to treat or prevent neuronal damage caused by disease or trauma, and wherein said polypeptide has at least one activity, such as, but not limited to, *in vitro* neurite outgrowth, *in vitro* neuroprotection, *in vivo* neuroprotection, and *in vivo* recuperation. These activities can be determined by known methods (Wagner, J. A., *et al.*, J. of Cell Biol., 103(4):1363-7 (1986); Lein eta al., Neuron, 15:597-605 (1995); Neufeld, G., *et al.*, J. of Cell. Phys., 131(1):131-40 (1987); Maiese, K., *et al.*, J. of Neurosci., 13(7):3034-40 (1993); Cheng, B., *et al.*, Neuron, 7(6):1031-41 (1991); Fisher, M., *et al.*, J. of Cereb. Blood Flow & Metabol., 15(6):953-9 (1995); Jiang, N., *et al.*, J. Neuro. Sci., 139(2):173-9 (1996); Kawamata, T., *et al.*, (1996); Kawamata, T., *et al.*, (1997a); Kawamata, T., Speliotes, E. K., and Finklestein, S. P. Adv. in Neurol., 73:377-82, (1997b)). An hFGF-8 polypeptide can thus be screened for a corresponding activity according to these and other known methods.

In another embodiment, the invention provides a method for inducing growth of a neuronal cell by contacting the cell with an amount of hFGF-8 polypeptide or an hFGF-8 encoding polynucleotide (in operable linkage with appropriate transcription elements) effective for inducing growth of the neuron either *in vitro* or *in vivo*.

A further embodiment of the present invention provides a method for inducing migration of a neuronal cell by contacting the cell with an amount of hFGF-8 polypeptide or an hFGF-8 encoding polynucleotide effective for inducing migration of the neuron.

The present invention also provides a method of treating and/or preventing neuronal damage caused by a disease or trauma wherein said method comprises administering to a patient a therapeutically effective amount of a composition comprising an isolated hFGF-8 nucleic acid and/or polypeptide as described herein and a carrier or diluent. The carrier or diluent can optionally be pharmaceutically acceptable, according to known methods.

In yet another embodiment, the invention provides a method for treating and/or preventing neuronal damage caused by disease or trauma wherein said method comprises administering a patient a therapeutically effective amount of an hFGF-8 polypeptide or an hFGF-8 encoding polynucleotide.

In another embodiment of the present invention said methods are used for treating or preventing neuronal damage caused by a disease or trauma wherein said disease or trauma includes, but is not limited to, trigeminal neuralgia, glossopharyngeal neuralgia, Bell's Palsy, myasthenia gravis, muscular dystrophy, muscle injury, progressive muscular atrophy, progressive bulbar inherited muscular atrophy, herniated, ruptured or prolapsed invertebrae disk, cervical spondylosis, plexus disorders, thoracic outlet destruction, peripheral neuropathy, such as those caused by lead, dapsone, ticks or porphyria, peripheral myelin disorders, Alzheimer's disease, Gullain-Barre syndrome, Parkinson's disease, Parkinsonian disorders, ALS, multiple sclerosis,

other central myelin disorders, stroke, ischemia associated with stroke, neural paropathy, other neural degenerative diseases, motor neuron diseases, sciatic crush, neuropathy associated with diabetes, spinal cord injuries, facial nerve crush, chemotherapy- or pharmacotherapy-induced neuropathy, and Huntington's disease.

The present invention provides methods for treating and/or preventing neuronal damage caused by disease or trauma comprising the administration of compositions comprising isolated, natural, recombinant, and/or synthetic nucleic acid molecules, or complements thereof, encoding at least one hFGF-8 polypeptide to a patient in need thereof. An hFGF-8 polypeptide of the invention comprises at least one fragment, domain, and/or variant as a portion or fragment of an hFGF-8 protein as described herein, having neuronal activity.

All publications or patents cited herein are entirely incorporated herein by reference as they show the state of the art at the time of the present invention to provide description and enablement of the present invention. Publications refer to scientific, patent publication or any other information available in any media format, including all recorded, electronic or printed formats. The following citations are entirely incorporated by reference: Ausubel, *et al.*, eds., Current Protocols in Molecular Biology, John Wiley & Sons, N.Y., N.Y. (1987-1998); Coligan, *et al.*, eds., Current Protocols in Protein Science, John Wiley & Sons, Inc., N.Y., N.Y. (1995-1999); Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor, N.Y. (1989); Harlow and Lane, Antibodies, a

Laboratory Manual, Cold Spring Harbor, N.Y. (1989); Coligan, *et al.*, eds., Current Protocols in Immunology, John Wiley & Sons, N.Y., N.Y. (1992-1999).

The following definitions of terms are intended to correspond to those as well known in the art. The following terms are therefore not limited to the definitions given, but are used according to the state of the art, as demonstrated by cited and/or contemporary publications or patents.

A " polynucleotide" comprises at least 10-20 nucleotides of a nucleic acid (RNA, DNA or combination thereof), provided by any means, such as synthetic, recombinant isolation or purification method steps.

The terms " complementary" or " complementarity" as used herein refer to the capacity of purine, pyrimidine, synthetic or modified nucleotides to associate by partial or complete complementarity through hydrogen or other bonding to form partial or complete double- or triple-stranded nucleic acid molecules. The following base pairs occur by complete complementarity: (i) guanine (G) and cytosine (C); (ii) adenine (A) and thymine (T); and adenine (A) and uracil (U). " Partial complementarity" refers to association of two or more bases by one or more hydrogen bonds or attraction that is less than the complete complementarity as described above. Partial or complete complementarity can occur between any two nucleotides, including naturally occurring or modified bases, e.g., as listed in 37 CFR § 1.822. All such nucleotides are included in polynucleotides of the invention as described herein.

The term " fusion protein" denotes a hybrid protein molecule not found in nature comprising a translational

fusion or enzymatic fusion in which two or more different proteins or fragments thereof are covalently linked on a single polypeptide chain. The term " polypeptide" also includes such fusion proteins.

The term " host cell" refers to any eucaryotic, procaryotic, or fusion or other cell or pseudo cell or membrane-containing construct that is suitable for propagating and/or expressing an isolated nucleic acid that is introduced into a host cell by any suitable means known in the art (e.g., but not limited to, transformation or transfection, or the like), or induced to express an endogenous nucleic acid encoding an hFGF-8 polypeptide according to the present invention. The cell can be part of a tissue or organism, isolated in culture or in any other suitable form.

The term " hybridization" as used herein refers to a process in which a partially or completely single-stranded nucleic acid molecule joins with a complementary strand through nucleotide base pairing. Hybridization can occur under conditions of low, moderate or high stringency, with high stringency preferred. The degree of hybridization depends upon, for example, the degree of homology, the stringency conditions, and the length of hybridizing strands as known in the art.

Reference to " isolated nucleic acid molecule(s)" intends a nucleic acid molecule, DNA, RNA, or both which has been removed from its native or naturally occurring environment. For example, recombinant nucleic acid molecules contained or generated in culture, a vector and/or a host cell are considered isolated for the purposes of the present invention. Further examples of isolated nucleic acid molecules include recombinant nucleic acid molecules

maintained in heterologous host cells or purified (partially or substantially) nucleic acid molecules in solution. Isolated RNA molecules include in vivo or in vitro RNA transcripts of the nucleic acid molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically, purified from or provided in cells containing such nucleic acids, where the nucleic acid exists in other than a naturally occurring form, quantitatively or qualitatively.

The term " isolated" used in reference to at least one polypeptide of the invention describes a state of isolation such that the peptide or polypeptide is not in a naturally occurring form and/or has been purified to remove at least some portion of cellular or non-cellular molecules with which the protein is naturally associated. However, " isolated" may include the addition of other functional or structural polypeptides for a specific purpose, where the other peptide may occur naturally associated with at least one polypeptide of the present invention, but for which the resulting compound or composition does not exist naturally.

The term " neurite outgrowth" includes dendritic and axonal outgrowth from neuronal cells. One of skill in the art will be familiar with the term neurite. More often, hFGF-8 activity is associated with dendritic outgrowth, and therefore the term dendritic growth or outgrowth is often used herein.

A " nucleic acid probe," " oligonucleotide probe," or " probe" as used herein comprises at least one detectably labeled or unlabeled nucleic acid which hybridizes under specified hybridization conditions with at least one other nucleic acid. This term also refers to a single- or

partially double-stranded nucleic acid, oligonucleotide or polynucleotide that will associate with a complementary or partially complementary target nucleic acid to form at least a partially double-stranded nucleic acid molecule. A nucleic acid probe may be an oligonucleotide or a nucleotide polymer. A probe can optionally contain a detectable moiety which may be attached to the end(s) of the probe or be internal to the sequence of the probe, termed a " detectable probe" or " detectable nucleic acid probe."

The term " parenteral" as used herein includes subcutaneous, intravenous, intramuscular, intra-articular, intra-synovial, intrasternal, intrathecal, intrahepatic, intralesional and intracranial injection or infusion techniques.

A " primer" is a nucleic acid fragment or oligonucleotide which functions as an initiating substrate for enzymatic or synthetic elongation of, for example, a nucleic acid molecule, e.g., using an amplification reaction, such as, but not limited to, a polymerase chain reaction (PCR), as known in the art.

The term " stringency" refers to hybridization conditions for nucleic acids in solution. High stringency conditions disfavor non-homologous base pairing. Low stringency conditions have much less of this effect. Stringency may be altered, for example, by changes in temperature and/or salt concentration, or other conditions, as well known in the art. A non-limiting example of " high stringency" conditions includes, for example, (a) a temperature of about 42°C , a formamide concentration of about $\leq$ 20%, and a low salt (SSC) concentration, or, alternatively, a temperature of about 65°C, or less, and a low salt (SSPE) concentration; (b) hybridization in 0.5 M

NaHPO4, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C (See, e.g., Ausubel, et al., ed., Current Protocols in Molecular Biology, 1987-1998, Wiley Interscience, New York, at §2.10.3). " SSC" comprises a hybridization and wash solution. A stock 20X SSC solution contains 3M sodium chloride, 0.3M sodium citrate, pH 7.0. " SSPE" comprises a hybridization and wash solution. A 1X SSPE solution contains 180 mM NaCl, 9mM Na2HPO4, 0.9 mM NaH2PO4 and 1 mM EDTA, pH 7.4.

The term " vector" as used herein refers to a nucleic acid compound used for introducing exogenous or endogenous nucleic acid into host cells. A vector comprises a nucleotide sequence which may encode one or more polypeptide molecules. Plasmids, cosmids, viruses and bacteriophages, in a natural state or which have undergone recombinant engineering, are non-limiting examples of commonly used vectors to provide recombinant vectors comprising at least one desired isolated nucleic acid molecule.

Nucleic Acid Molecules

Using the information provided herein, such as the nucleotide sequences encoding at least 90-100% of the contiguous amino acids of at least one of SEQ ID NOS:4, 5, 6, 7, specified fragments or variants thereof, or a deposited vector comprising at least one of these sequences, a nucleic acid molecule of the present invention encoding an hFGF-8 polypeptide can be obtained using well-known methods.

Nucleic acid molecules used in the present invention can be in the form of RNA, such as mRNA, hnRNA, tRNA or any other form, or in the form of DNA, including, but not limited to, cDNA and genomic DNA obtained by cloning or produced synthetically, or any combination thereof. The DNA

can be triple-stranded, double-stranded or single-stranded, or any combination thereof. Any portion of at least one strand of the DNA or RNA can be the coding strand, also known as the sense strand, or it can be the non-coding strand, also referred to as the anti-sense strand.

Isolated nucleic acid molecules used in the present invention include nucleic acid molecules comprising an open reading frame (ORF) shown in at least one of SEQ ID NOS:1, 2, 3, and 8; nucleic acid molecules comprising the coding sequence for an hFGF-8 polypeptide; and nucleic acid molecules which comprise a nucleotide sequence substantially different from those described above but which, due to the degeneracy of the genetic code, still encode at least one hFGF-8 polypeptide as described herein. Of course, the genetic code is well known in the art. Thus, it would be routine for one skilled in the art to generate such degenerate nucleic acid variants that code for specific hFGF-8 polypeptides of the present invention. See, e.g., Ausubel, *et al.*, *supra*, and such nucleic acid variants are included in the present invention.

The nucleic acid molecules necessary to practice an embodiment of the present invention are provided encoding the mature hFGF-8 polypeptide or the full-length hFGF-8 polypeptide lacking the N-terminal methionine. The invention can also utilize an isolated nucleic acid molecule having the nucleotide sequence shown in at least one of SEQ ID NOS:1, 2, 3, and 8, or the nucleotide sequence of the hFGF-8 cDNA contained in at least one of the above-described deposited clones listed herein, or a nucleic acid molecule having a sequence complementary thereto. Such isolated molecules, particularly nucleic acid molecules, are useful as probes for gene mapping by in situ hybridization with

chromosomes, and for detecting transcription, translation and/or expression of the hFGF-8 gene in human tissue, for instance, by Northern blot analysis for mRNA detection.

Unless otherwise indicated, all nucleotide sequences identified by sequencing a nucleic acid molecule herein can be or were identified using an automated nucleic acid sequencer, and all amino acid sequences of polypeptides encoded by nucleic acid molecules identified herein can be or were identified by codon correspondence or by translation of a nucleic acid sequence identified using method steps as described herein or as known in the art. Therefore, as is well known in the art that for any nucleic acid sequence identified by this automated approach, any nucleotide sequence identified herein may contain some errors which are reproducibly correctable by resequencing based upon an available or a deposited vector or host cell containing the nucleic acid molecule using known methods.

Nucleotide sequences identified by automation are typically at least about 95% to at least about 99.999% identical to the actual nucleotide sequence of the sequenced nucleic acid molecule. The actual sequence can be more precisely identified by other approaches including manual nucleic acid sequencing methods well known in the art. As is also known in the art, a single insertion or deletion in an identified nucleotide sequence compared to the actual sequence will cause a frame shift in translation of the nucleotide sequence such that the identified amino acid sequence encoded by an identified nucleotide sequence will be completely different from the amino acid sequence actually encoded by the sequenced nucleic acid molecule, beginning at the point of such an insertion or deletion.

Nucleic Acid Fragments

The present invention can further depend upon fragments of the isolated nucleic acid molecules described herein. By a fragment of an isolated nucleic acid molecule is meant a molecule having at least 10 nucleotides of a nucleotide sequence of a deposited cDNA or a nucleotide sequence shown in at least one of SEQ ID NOS:1, 2, 3, and 8, and is intended to mean fragments at least about 10 nucleotides, and at least about 40 nucleotides in length, which are useful, *inter alia* as diagnostic probes and primers as described herein. Of course, larger fragments such as at least about 50, 100, 120, 200, 500, 1000, 1500, 2000, 2500, 3000, 3500, and/or 4000 or more nucleotides in length, are also useful according to the present invention as are fragments corresponding to most, if not all, of the nucleotide sequence (or the deposited cDNA) as shown at least one of SEQ ID NOS:1, 2, 3, and 8. By a fragment at least 10 nucleotides in length, for example, is intended fragments which include 10 or more contiguous nucleotides from the nucleotide sequence of the deposited cDNA or the nucleotide sequence as shown in SEQ ID NOS:1, 2, 3, 8, or consensus sequences thereof, as determined by methods known in the art (See e.g., Ausubel, *supra*, Chapter 7).

Such nucleotide fragments are useful according to the present invention for screening DNA sequences that code for one or more fragments of an hFGF-8 polypeptide as described herein. Such screening, as a non-limiting example can include the use of so-called " DNA chips" for screening DNA sequences of the present invention of varying lengths, as described, e.g., in U.S. Patent Nos. 5,631,734, 5,624,711, 5,744,305, 5,770,456, 5,770,722, 5,675,443, 5,695,940,

5,710,000, 5,733,729, which are entirely incorporated herein by reference.

As indicated, nucleic acid molecules useful according to the present invention may comprise a nucleic acid encoding an hFGF-8 polypeptide including, but not limited to, those encoding the amino acid sequence of the mature polypeptide, by itself; the coding sequence for the mature polypeptide and additional sequences, such as the coding sequence of at least one signal leader or fusion peptide or of the mature polypeptide, with or without the aforementioned additional coding sequences, such as at least one intron, together with additional, non-coding sequences, including but not limited to, introns and non-coding 5' and 3' sequences, such as the transcribed, non-translated sequences that play a role in transcription, mRNA processing, including splicing and polyadenylation signals (for example - ribosome binding and stability of mRNA); an additional coding sequence which codes for additional amino acids, such as those which provide additional functionalities. Thus, the sequence encoding a polypeptide can be fused to a marker sequence, such as a sequence encoding a peptide that facilitates purification of the fused polypeptide.

Oligonucleotide and Polynucleotide Probes and/or Primers

In another aspect, the invention may use a polynucleotide (either DNA or RNA) that comprises at least about 20 nt, still more preferably at least about 30 nt, and even more preferably at least about 30-2000 nt of a nucleic acid molecule described herein. These are useful as diagnostic probes and primers as discussed above and in more detail below.

By a portion of a polynucleotide of " at least 10 nt in length," for example, is intended 10 or more contiguous nucleotides from the nucleotide sequence of the reference polynucleotide (e.g., at least one deposited nucleic acid or at least one nucleotide sequence as shown in at least one of SEQ ID NOS:1, 2, 3, and 8).

Of course, a polynucleotide which hybridizes only to a poly A sequence (such as the 3' terminal poly(A) of an hFGF-8 cDNA shown in at least one of SEQ ID NOS:1, 2, 3, 8, or to a complementary stretch of T (or U) resides, would not be included in a probe of the invention, since such a polynucleotide would hybridize to any nucleic acid molecule containing a poly (A) stretch or the complement thereof (e.g., practically any double-stranded cDNA clone).

The present invention may also utilize subsequences of full-length nucleic acids. Any number of subsequences can be obtained by reference to at least one of SEQ ID NOS:1, 2, 3, 8, or a complementary sequence, and using primers which selectively amplify, under stringent conditions to: at least two sites to the polynucleotides of the present invention, or to two sites within the nucleic acid which flank and comprise a polynucleotide of the present invention, or to a site within a polynucleotide of the present invention and a site within the nucleic acid which comprises it. A variety of methods for obtaining 5' and/or 3' ends is well known in the art. See, e.g., RACE (Rapid Amplification of Complementary Ends) as described in M. A. Frohman, PCR Protocols: A Guide to Methods and Applications, M. A. Innis, *et al*, Eds., Academic Press, Inc., San Diego, CA, pp. 28-38 (1990); see also, U.S. Patent No. 5,470,722, and Ausubel, *et al.*, Current Protocols in Molecular Biology, Chapter 15, Eds., John Wiley & Sons, N.Y. (1989-1999). Thus, the present invention

provides hFGF-8 polynucleotides having the sequence of the hFGF-8 gene, nuclear transcript, cDNA, or complementary sequences and/or subsequences thereof.

Primer sequences can be obtained by reference to a contiguous subsequence of a polynucleotide of the present invention. Primers are chosen to selectively hybridize, under PCR amplification conditions, to a polynucleotide of the present invention in an amplification mixture comprising a genomic and/or cDNA library from the same species. Generally, the primers are complementary to a subsequence of the amplified nucleic acid. In some embodiments, the primers will be constructed to anneal at their 5′ terminal ends to the codon encoding the carboxy or amino terminal amino acid residue (or the complements thereof) of the polynucleotides of the present invention. The primer length in nucleotides is selected from the group of integers consisting of from at least 15 to 50. Thus, the primers can be at least 15, 18, 20, 25, 30, 40, or 50 nucleotides in length or any range or value therein. A non-annealing sequence at the 5′ end of the primer (a " tail") can be added, for example, to introduce a cloning site at the terminal ends of the amplified DNA.

The amplification primers may optionally be elongated in the 3′ direction with additional contiguous or complementary nucleotides from the polynucleotide sequences, such as at least one of SEQ ID NOS:1, 2, 3, and 8, from which they are derived. The number of nucleotides by which the primers can be elongated is selected from the group of integers consisting of from at least 1 to at least 25. Thus, for example, the primers can be elongated with an additional 1, 5, 10, or 15 nucleotides or any range or value therein. Those of skill will recognize that a lengthened primer sequence can be employed to increase specificity of binding

(i.e., annealing) to a target sequence, or to add useful sequences, such as links or restriction sites (See e.g., Ausubel, *supra*, Chapter 15).

The amplification products can be translated using expression systems well known to those of skill in the art and as discussed, infra. The resulting translation products can be confirmed as polypeptides of the present invention by, for example, assaying for the appropriate catalytic activity (e.g., specific activity and/or substrate specificity), or verifying the presence of one or more linear epitopes which are specific to a polypeptide of the present invention. Methods for protein synthesis from PCR derived templates are known in the art (See e.g., Ausubel, *supra*, Chapters 9, 10, 15; Coligan, Current Protocols in Protein Science, *supra*, Chapter 5) and available commercially. See, e.g., Amersham Life Sciences, Inc., Catalog '97, p. 354.

Polynucleotides Which Selectively Hybridize to a Polynucleotide as Described Herein

The present invention may also utilize isolated nucleic acids that hybridize under selective hybridization conditions to a polynucleotides disclosed herein, e.g., SEQ ID NOS:1, 2, 3, and 8. Thus, the polynucleotides of this embodiment can be used for isolating, detecting, and/or quantifying nucleic acids comprising such polynucleotides. For example, polynucleotides used in the present invention can be used to identify, isolate, or amplify partial or full-length clones in a deposited library. The polynucleotides may be genomic or cDNA sequences isolated, or otherwise complementary to, a cDNA from a human or mammalian nucleic acid library. Preferably, the cDNA library comprises at least 80% full-length sequences, preferably at least 85% or 90% full-length sequences, and more preferably at least 95% full-length

sequences. The cDNA libraries can be normalized to increase the representation of rare sequences. Low stringency hybridization conditions are typically, but not exclusively, employed with sequences having a reduced sequence identity relative to complementary sequences. Moderate and high stringency conditions can optionally be employed for sequences of greater identity. Low stringency conditions allow selective hybridization of sequences having about 70% sequence identity and can be employed to identify orthologous or paralogous sequences.

Polynucleotides Complementary to the Polynucleotides

As indicated above, embodiments of the present invention make use of isolated nucleic acids comprising hFGF-8 polynucleotides, wherein the polynucleotides are complementary to the polynucleotides described herein, above. As those of skill in the art will recognize, complementary sequences base pair throughout the entirety of their length with such polynucleotides (i.e., have 100% sequence identity over their entire length). Complementary bases associate through hydrogen bonding in double-stranded nucleic acids. For example, the following base pairs are complementary: guanine and cytosine; adenine and thymine; and adenine and uracil (See, e.g., Ausubel, *supra*, Chapter 67; or Sambrook, *supra*).

Construction of Nucleic Acids

The isolated nucleic acids useful in the present invention can be made using (a) standard recombinant methods, (b) synthetic techniques, (c) purification techniques, or combinations thereof, as well known in the art.

The nucleic acids may conveniently comprise sequences in addition to a polynucleotide of the present invention. For example, a multi-cloning site comprising one or more endonuclease restriction sites may be inserted into the nucleic acid to aid in isolation of the polynucleotide. Also, translatable sequences may be inserted to aid in the isolation of the translated polynucleotide of the present invention. For example, a hexa-histidine marker sequence provides a convenient means to purify the proteins of the present invention. The nucleic acid of the present invention - excluding the polynucleotide sequence - is optionally a vector, adapter, or linker for cloning and/or expression of a polynucleotide of the present invention.

Additional sequences may be added to such cloning and/or expression sequences to optimize their function in cloning and/or expression, to aid in isolation of the polynucleotide, or to improve the introduction of the polynucleotide into a cell. Typically, the length of a nucleic acid of the present invention less the length of its polynucleotide of the present invention is less than 20 kilobase pairs, often less than 15 kb, and frequently less than 10 kb. Use of cloning vectors, expression vectors, adapters, and linkers is well known in the art (See, e.g., Ausubel, *supra*, Chapters 1-5; or Sambrook, *supra*).

Recombinant Methods for Constructing Nucleic Acids

The isolated nucleic acid compositions of this invention, such as RNA, cDNA, genomic DNA, or a hybrid thereof, can be obtained from biological sources using any number of cloning methodologies known to those of skill in the art. In some embodiments, oligonucleotide probes that selectively hybridize, under stringent conditions, to the

polynucleotides of the present invention are used to identify the desired sequence in a cDNA or genomic DNA library. While isolation of RNA, and construction of cDNA and genomic libraries is well known to those of ordinary skill in the art (See, e.g., Ausubel, *supra*, Chapters 1-7; or Sambrook, *supra*).

Nucleic Acid Screening and Isolation Methods

A cDNA or genomic library can be screened using a probe based upon the sequence of a polynucleotide of the present invention, such as those disclosed herein. Probes may be used to hybridize with genomic DNA or cDNA sequences to isolate homologous genes in the same or different organisms. Those of skill in the art will appreciate that various degrees of stringency of hybridization can be employed in the assay; and either the hybridization or the wash medium can be stringent. As the conditions for hybridization become more stringent, there must be a greater degree of complementarity between the probe and the target for duplex formation to occur. Temperature, ionic strength, pH and the presence of a partially denaturing solvent such as formamide can control the degree of stringency. Changing the polarity of the reactant solution through, for example, manipulation of the concentration of formamide within the range of 0% to 50% conveniently varies the stringency of hybridization. The degree of complementarity (sequence identity) required for detectable binding will vary in accordance with the stringency of the hybridization medium and/or wash medium. The degree of complementarity will optimally be 100%; however, it should be understood that minor sequence variations in the probes and primers may be compensated for

by reducing the stringency of the hybridization and/or wash medium.

Methods of amplification of RNA or DNA are well known in the art and can be used according to the present invention without undue experimentation, based on the teaching and guidance presented herein.

Known methods of DNA or RNA amplification include, but are not limited to, polymerase chain reaction (PCR) and related amplification processes (see, e.g., U.S. Patent Nos. 4,683,195, 4,683,202, 4,800,159, 4,965,188, to Mullis, *et al.*; 4,795,699 and 4,921,794 to Tabor, et al; 5,142,033 to Innis; 5,122,464 to Wilson, *et al.*; 5,091,310 to Innis; 5,066,584 to Gyllensten, *et al*; 4,889,818 to Gelfand, *et al*; 4,994,370 to Silver, *et al*; 4,766,067 to Biswas; 4,656,134 to Ringold) and RNA mediated amplification which uses anti-sense RNA to the target sequence as a template for double-stranded DNA synthesis (U.S. Patent No. 5,130,238 to Malek, *et al*, with the tradename NASBA), the entire contents of which are herein incorporated by reference (See, e.g., Ausubel, *supra*, Chapter 15; or Sambrook, *supra*). For instance, polymerase chain reaction (PCR) technology can be used to amplify the sequences of polynucleotides of the present invention and related genes directly from genomic DNA or cDNA libraries. PCR and other in vitro amplification methods may also be useful, for example, to clone nucleic acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of the desired mRNA in samples, for nucleic acid sequencing, or for other purposes. Examples of techniques sufficient to direct persons of skill through *in vitro* amplification methods are found in Berger, Sambrook, and Ausubel (e.g., Chapter 15) *supra*, as well as Mullis, *et al.*,

U.S. Patent No. 4,683,202 (1987); and Innis, *et al.*, PCR Protocols A Guide to Methods and Applications, Eds., Academic Press Inc., San Diego, CA (1990). Commercially available kits for genomic PCR amplification are known in the art. See, e.g., Advantage-GC Genomic PCR Kit (Clontech). The T4 gene 32 protein (Boehringer Mannheim) can be used to improve yield of long PCR products.

Synthetic Methods for Constructing Nucleic Acids

The isolated nucleic acids required to practice the present invention can also be prepared by direct chemical synthesis by methods such as the phosphotriester method of Narang, et al., Meth. Enzymol. 68:90-99 (1979); the phosphodiester method of Brown, *et al.*, Meth. Enzymol. 68:109-151 (1979); the diethylphosphoramidite method of Beaucage, *et al.*, Tetra. Letts. 22:1859-1862 (1981); the solid phase phosphoramidite triester method described by Beaucage and Caruthers, Tetra. Letts. 22(20):1859-1862 (1981), e.g., using an automated synthesizer, e.g., as described in Needham-VanDevanter, *et al.*, Nucleic Acids Res. 12:6159-6168 (1984); and the solid support method of U.S. Patent No. 4,458,066. Chemical synthesis generally produces a single-stranded oligonucleotide, which may be converted into double-stranded DNA by hybridization with a complementary sequence, or by polymerization with a DNA polymerase using the single strand as a template. One of skill in the art will recognize that while chemical synthesis of DNA can be limited to sequences of about 100 or more bases,, longer sequences may be obtained by the ligation of shorter sequences.

Recombinant Expression Cassettes

The present invention can be practiced using recombinant expression cassettes comprising an hFGF-8 nucleic acid. A nucleic acid sequence useful in the present invention, for example a cDNA or a genomic sequence encoding a full-length polypeptide of the present invention, can be used to construct a recombinant expression cassette which can be introduced into at least one desired host cell. A recombinant expression cassette will typically comprise a polynucleotide operably linked to transcriptional initiation regulatory sequences that will direct the transcription of the polynucleotide in the intended host cell.

Both heterologous and non-heterologous (i.e., endogenous) promoters can be employed to direct expression of the nucleic acids comprising the present invention. These promoters can also be used, for example, in recombinant expression cassettes to drive expression of antisense nucleic acids to reduce, increase, or alter hFGF-8 content and/or composition in a desired tissue.

In some embodiments, isolated nucleic acids which serve as promoter or enhancer elements can be introduced in the appropriate position (generally upstream) of a non-heterologous form of a polynucleotide of the present invention so as to up or down regulate expression of a polynucleotide of the present invention. For example, endogenous promoters can be altered *in vivo* or *in vitro* by mutation, deletion and/or substitution.

A polynucleotide can be expressed in either sense or anti-sense orientation as desired. It will be appreciated that control of gene expression in either sense or anti-sense orientation can have a direct impact on the observable characteristics.

Another method of suppression is sense suppression. Introduction of nucleic acid configured in the sense orientation has been shown to be an effective means by which to block the transcription of target genes.

A variety of cross-linking agents, alkylating agents and radical generating species as pendant groups on polynucleotides of the present invention can be used to bind, label, detect and/or cleave nucleic acids. Knorre, *et al.*, Biochimie 67:785-789 (1985); Vlassov, *et al.*, Nucleic Acids Res. 14:4065-4076 (1986); Iverson and Dervan, J. Am. Chem. Soc. 109:1241-1243 (1987); Meyer, *et al.*, J. Am. Chem. Soc. 111:8517-8519 (1989); Lee, *et al.*, Biochemistry 27:3197-3203 (1988); Home, *et al.*, J. Am. Chem. Soc. 112:2435-2437 (1990); Webb and Matteucci, J. Am. Chem. Soc. 108:2764-2765 (1986); Nucleic Acids Res. 14:7661-7674 (1986); Feteritz, *et al.*, J. Am. Chem. Soc. 113:4000 (1991). Various compounds to bind, detect, label, and/or cleave nucleic acids are known in the art. See, for example, U.S. Patent Nos. 5,543,507; 5,672,593; 5,484,908; 5,256,648; and 5,681941, each entirely incorporated herein by reference.

Vectors and Host cells

The present invention also relates to vectors that include isolated nucleic acid molecules of the present invention, host cells that are genetically engineered with the recombinant vectors, and the production of hFGF-8 polypeptides or fragments thereof by recombinant techniques, as is well known in the art. See, e.g., Sambrook, *et al.*, *supra*; Ausubel, *supra*, Chapters 1-9, each entirely incorporated herein by reference.

The polynucleotides can optionally be joined to a vector containing a selectable marker for propagation in a

host. Generally, a plasmid vector is introduced in a precipitate, such as a calcium phosphate precipitate, or in a complex with a charged lipid. If the vector is a virus, it can be packaged in vitro using an appropriate packaging cell line and then transduced into host cells.

The DNA insert should be operatively linked to an appropriate promoter, such as the phage lambda PL promoter, the *E. coli lac*, *trp* and *tac* promoters, the SV40 early and late promoters and promoters of retroviral LTRs, or any other suitable promoter. The skilled artisan will know other suitable promoters. The expression constructs will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome-binding site for translation. The coding portion of the mature transcripts expressed by the constructs will preferably include a translation initiating at the beginning and a termination codon (e.g., UAA, UGA or UAG) appropriately positioned at the end of the mRNA to be translated, with VAA and VAG preferred for mammalian or eukaryotic cell expression.

Expression vectors will preferably include at least one selectable marker. Such markers include, e.g., ampicillin, dihydrofolate reductase, hygromycin, or neomycin (G418) resistance for eukaryotic cell culture, and tetracycline or ampicillin resistance genes for culturing in E. coli and other bacteria or prokaryotics. Representative examples of appropriate hosts include, but are not limited to, bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells; insect cells such as Drosophila S2 and Spodoptera Sf9 cells; animal cells such as CHO, COS and Bowes melanoma cells; and plant cells. Appropriate culture mediums and conditions for the

above-described host cells are known in the art. Vectors preferred for use in bacteria include pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Preferred eucaryotic vectors include pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan. See, e.g., Ausubel, *supra*, Chapter 1; Coligan, Current Protocols in Protein Science, *supra*, Chapter 5.

Introduction of a vector construct into a host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as Sambrook, *supra*, Chapters 1-4 and 16-18; Ausubel, *supra*, Chapters 1, 9, 13, 15, 16.

Polypeptide(s) required to practice the present invention can be expressed in a modified form, such as a fusion protein, and can include not only secretion signals, but also additional heterologous functional regions. For instance, a region of additional amino acids, particularly charged amino acids, can be added to the N-terminus of a polypeptide to improve stability and persistence in the host cell, during purification, or during subsequent handling and storage. Also, peptide moieties can be added to a polypeptide to facilitate purification. Such regions can be removed prior to final preparation of a polypeptide. Such methods are described in many standard laboratory manuals,

such as Sambrook, *supra*, Chapters 17 and 18; Ausubel, *supra*, Chapters 16, 17 and 18.

Expression of Proteins in Host Cells

Using nucleic acids, one may express a protein of the used in the present invention in a recombinantly engineered cell, such as bacteria, yeast, insect, or mammalian cells. The cells produce the protein in a non-natural condition (e.g., in quantity, composition, location, and/or time), because they have been genetically altered through human intervention to do so.

It is expected that those of skill in the art are knowledgeable in the numerous expression systems available for expression of a nucleic acid encoding a protein of the present invention. No attempt to describe in detail the various methods known for the expression of proteins in prokaryotes or eukaryotes will be made.

In brief summary, the expression of isolated nucleic acids encoding a protein according to the present invention will typically be achieved by operably linking, for example, the DNA or cDNA to a promoter (which is either constitutive or inducible) followed by incorporation into an expression vector. The vectors can be suitable for replication and integration in either prokaryotes or eukaryotes. Typical expression vectors contain transcription and translation terminators, initiation sequences and promoters useful for regulation of the expression of the DNA encoding a protein of the present invention. To obtain high level expression of a cloned gene, it is desirable to construct expression vectors which contain, at the minimum, a strong promoter to direct transcription, a ribosome binding site for translational initiation, and a transcription/translation terminator. One

of skill would recognize that modifications can be made to a protein of the present invention without diminishing its biological activity.

Some modifications may be made to facilitate the cloning, expression, or incorporation of the targeting molecule into a fusion protein. Such modifications are well known to those of skill in the art and include, for example, a methionine added at the amino terminus to provide an initiation site, or additional amino acids (e.g., poly His) placed on either terminus to create conveniently located restriction sites, termination codons, or purification sequences.

Alternatively, to practice the present invention hFGF-8 nucleic acids can be expressed in a host cell by turning on (by manipulation) in a host cell that contains endogenous DNA encoding a polypeptide of the present invention. Such methods are well known in the art, e.g., as described in US patent Nos. 5,580,734, 5,641,670, 5,733,746, and 5,733,761, entirely incorporated herein by reference.

Expression in Prokaryotes

Prokaryotic cells may be used as hosts for expression. Prokaryotes most frequently are represented by various strains of E. coli; however, other microbial strains may also be used. Commonly used prokaryotic control sequences which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences, include such commonly used promoters as the beta lactamase (penicillinase) and lactose (lac) promoter systems (Chang, *et al.*, Nature 198:1056 (1977)), the tryptophan (trp) promoter system (Goeddel, *et al.*, Nucleic Acids Res. 8:4057 (1980)) and the lambda derived P L promoter

and N-gene ribosome binding site (Shimatake, *et al.*, Nature 292:128 (1981)). The inclusion of selection markers in DNA vectors transfected in E. coli is also useful. Examples of such markers include genes specifying resistance to ampicillin, tetracycline, or chloramphenicol.

The vector is selected to allow introduction into the appropriate host cell. Bacterial vectors are typically of plasmid or phage origin. Appropriate bacterial cells are infected with phage vector particles or transfected with naked phage vector DNA. If a plasmid vector is used, the bacterial cells are transformed with the plasmid vector DNA. Expression systems for expressing a protein of the present invention are available using Bacillus sp. and Salmonella (Palva, *et al.*, Gene 22:229-235 (1983); Mosbach, *et al.*, Nature 302:543-545 (1983)). See, e.g., Ausubel, *supra*, Chapters 1-3, 16(Sec.1); and Coligan, *supra*, Current Protocols in Protein Science, Units 5.1, 6.1-6.7.

Expression in Eukaryotes

A variety of eukaryotic expression systems such as yeast, insect cell lines, plant and mammalian cells, are known to those of skill in the art. As explained briefly below, a nucleic acid useful in the present invention can be expressed in these eukaryotic systems.

Synthesis of heterologous proteins in yeast is well known. F. Sherman, *et al.*, Methods in Yeast Genetics, Cold Spring Harbor Laboratory (1982) is a well-recognized work describing the various methods available to produce the protein in yeast. Two widely utilized yeast for production of eukaryotic proteins are Saccharomyces cerevisiae and Pichia pastoris. Vectors, strains, and protocols for

expression in Saccharomyces and Pichia are known in the art and available from commercial suppliers (e.g., Invitrogen). Suitable vectors usually have expression control sequences, such as promoters, including 3-phosphoglycerate kinase or alcohol oxidase, and an origin of replication, termination sequences and the like as desired.

A hFGF-8 protein, once expressed, can be isolated from yeast by lysing the cells and applying standard protein isolation techniques to the lysates. The monitoring of the purification process can be accomplished by using Western blot techniques or radioimmunoassay of other standard immunoassay techniques.

The sequences encoding proteins comprising the present invention can also be ligated to various expression vectors for use in transfecting cell cultures of, for instance, mammalian, insect, or plant origin. Illustrative of cell cultures useful for the production of the peptides are mammalian cells. Mammalian cell systems often will be in the form of monolayers of cells although mammalian cell suspensions may also be used. A number of suitable host cell lines capable of expressing intact proteins have been developed in the art, and include the HEK293, BHK21, and CHO cell lines. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter (e.g., the CMV promoter, a HSV tk promoter or pgk (phosphoglycerate kinase) promoter), an enhancer (Queen, *et al.*, Immunol. Rev. 89:49 (1986)), and processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites (e.g., an SV40 large T Ag poly A addition site), and transcriptional terminator sequences. Other animal cells useful for production of proteins of the present invention are available, for

instance, from the American Type Culture Collection Catalogue of Cell Lines and Hybridomas (7th edition, 1992).

Appropriate vectors for expressing proteins used in the present invention in insect cells are usually derived from the SF9 baculovirus. Suitable insect cell lines include mosquito larvae, silkworm, armyworm, moth and Drosophila cell lines such as a Schneider cell line (See Schneider, J. Embryol. Exp. Morphol. 27:353-365 (1987).

As with yeast, when higher animal or plant host cells are employed, polyadenlyation or transcription terminator sequences are typically incorporated into the vector. An example of a terminator sequence is the polyadenlyation sequence from the bovine growth hormone gene. Sequences for accurate splicing of the transcript may also be included. An example of a splicing sequence is the VP1 intron from SV40 (Sprague, *et al.*, J. Virol. 45:773-781 (1983)). Additionally, gene sequences to control replication in the host cell may be incorporated into the vector such as those found in bovine papilloma virus type-vectors. M. Saveria-Campo, Bovine Papilloma Virus DNA, a Eukaryotic Cloning Vector in DNA Cloning Vol. II, a Practical Approach, D. M. Glover, Ed., IRL Press, Arlington, VA, pp. 213-238 (1985).

Protein Purification

An hFGF-8 polypeptide can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography (" HPLC") is

employed for purification. Polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eucaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention can be glycosylated or can be non-glycosylated. In addition, polypeptides of the invention can also include an initial modified methionine residue, in some cases as a result of host-mediated processes. Such methods are described in many standard laboratory manuals, such as Sambrook, supra, Chapters 17.37-17.42; Ausubel, supra, Chapters 10, 12, 13, 16, 18 and 20.

hFGF-8 polypeptides, fragments, and variants

The invention may furthermore use isolated hFGF-8 polypeptides having fragments or specified variants of the amino acid sequence encoded by the deposited cDNAs, or the amino acid sequence in SEQ ID NOS:4, 5, 6, and 7.

The isolated proteins used in the present invention comprise a polypeptide encoded by any one of the polynucleotides of the present invention as discussed more fully, supra, or polypeptides which are fragments or variants thereof.

The present invention provides compositions comprising hFGF-8 polypeptides as described herein which possess neuroprotective, neuronal recovery, neural migration inducing, or dendritic or axonal growth promoting effects on neurons. Exemplary polypeptide sequences are provided in SEQ ID NOS:4, 5, 6, and 7. To practice the present invention hFGF-8 proteins or variants thereof can comprise any number

of contiguous amino acid residues from an hFGF-8 polypeptide, wherein that number is selected from the group of integers consisting of from 90-100% of the number of contiguous residues in a full-length hFGF-8 polypeptide. Optionally, this subsequence of contiguous amino acids is at least 50, 60, 70, 80, or 90 amino acids in length. Further, the number of such subsequences can be any integer selected from the group consisting of from 1 to 20, such as 2, 3, 4, or 5.

Further, an hFGF-8 polypeptide component of the present invention can include one or more amino acid substitutions, deletions or additions, either from natural mutations or human manipulation, as specified herein.

Of course, the number of amino acid substitutions a skilled artisan would make depends on many factors, including those described above. Generally speaking, the number of amino acid substitutions, insertions or deletions for any given hFGF-8 polypeptide will not be more than 40, 30, 20, 10, 5, or 3, such as 1-30 or any range or value therein, as specified herein.

Amino acids in an hFGF-8 polypeptide that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, Science 244:1081-1085 (1989)). The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for biological activity. Sites that are critical for ligand-protein binding can also be identified by structural analysis such as crystallization, nuclear magnetic resonance or photoaffinity labeling (Smith, *et al.*, J. Mol. Biol. 224:899-904 (1992) and de Vos, *et al.*, Science 255:306-312 (1992)).

hFGF-8 polypeptides required to practice the present invention can include but are not limited to, at least one selected from SEQ ID NOS:4, 5, 6, and 7.

An hFGF-8 polypeptide can further comprise a polypeptide of at least one of 204, 215, 233, and 244, contiguous amino acids of SEQ ID NOS:4, 5, 6, and 7, respectively.

An hFGF-8 polypeptide further includes an amino acid sequence selected from one or more of SEQ ID NOS:4, 5, 6, and 7.

Non-limiting mutants that can enhance or maintain at least one of the listed activities include, but are not limited to, any of the above polypeptides, further comprising at least one mutation corresponding to at least one substitution, insertion or deletion of SEQ ID NOS:4, 5, 6, and 7.

The present invention also provides a composition comprising an isolated hFGF-8 nucleic acid and/or polypeptide as described herein, and a carrier or diluent.

According to another embodiment, the invention provides methods for stimulating nerve growth, neurite outgrowth, neuronal migration, recovery of function, and/or preventing neurodegeneration. In one aspect of this embodiment, the method used to stimulate nerve growth, neurite outgrowth, neuronal migration, recovery of function, and/or preventing neurodegeneration comprises administering to a non-human animal a pharmaceutically acceptable composition comprising any of the hFGF-8 compounds described herein and a pharmaceutically acceptable carrier. The amount of compound utilized in these methods is between about 0.01 and 10 mg/kg body weight/day.

The present invention also provides a composition comprising an isolated hFGF-8 nucleic acid and/or polypeptide as described herein, at least one other neurotrophic, neuroprotective, thrombolytic, and/or anti-thrombotic agent, and a carrier or diluent. The other neurotrophic, neuroprotective, thrombolytic, and/or anti-thrombotic agents may include, but are not limited to, nerve growth factor (NGF), insulin growth factor (IGF-1) and its active truncated derivatives such as gIGF-1, acidic and basic fibroblast growth factor (aFGF and bFGF, respectively), platelet-derived growth factors (PDGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factors (CNTF), glial cell line-derived neurotrophic factor (GDNF), neurotrophin-3 (NT-31, neurotrophin 4/S (NT-4/S), or any of the compounds described in WO 97/36869, WO 96/41609, WO 97/16190, WO96/40633, WO 97/18828, WO 96/40140 or WO 98/13355. The most preferred neurotrophic agent in the compositions of the present invention is NGF.

According to another embodiment, this invention provides methods for stimulating nerve growth, neurite outgrowth, neuronal migration, neuronal recovery of function, and/or preventing neurodegeneration in a patient in need thereof. In one aspect of this embodiment, said method comprises administering to the patient a pharmaceutically acceptable composition comprising any of the hFGF-8 compounds of this invention in conjunction with at least one other neurotrophic, neuroprotective, thrombolytic, and/or anti-thrombotic agent, and a pharmaceutically acceptable carrier.

The pharmaceutical compositions of the present invention maybe administered orally, parenterally, by

inhalationspray topically, rectally, nasally, buccally, vaginally or via an implanted reservoir. Preferably, the compositions are administered orally, intraperitoneally or intravenously. The pharmaceutical compositions of this invention may contain any conventional non-toxic pharmaceutically-acceptable carriers, adjuvants or vehicles. In some cases, the pH of the formulation maybe adjusted with pharmaceutically acceptable acids, bases or buffers to enhance the stability of the formulated compound or its delivery form. Sterile injectable forms of the compositions of this invention may be aqueous or oleaginous suspension. These suspensions may be formulated according to techniques known in the art using suitable dispersion wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium.

For this purpose, any bland fixed oil may be employed including synthetic mono or di-glycerides. Fatty acids, such as oleic acid and its glyceride derivatives are useful in the preparation of injectables, as are natural pharmaceutically acceptable oils, such as olive oil or castor oil, especially in their polyoxyethylated versions. These oil solutions or suspensions may also contain a long-chain alcohol diluent or dispersant, such as Ph. Hely or similar alcohol.

The pharmaceutical compositions of this invention may be orally administered in any orally acceptable dosage form

including, but not limited to, capsules, tablets, aqueous suspensions or solutions. In the case of tablets for oral use, carriers which are commonly used include lactose, corn starch, dicalcium phosphate and microcrystalline cellulose (Avicel). Lubricating agents, such as magnesium stearate and talc, are also typically added. For oral administration in a capsule form, useful diluents include lactose, dried corn starch and TPGS,as well as the other diluents used in tablets. For oral administration in a soft gelatincapsule form (filled with either a suspension or a solution of a compound of this invention), useful diluents include PEG400, TPGS, propylene glycol, Labrasol, Gelucire, Transcutol, PVP and potassium acetate. When aqueous suspensions are administered orally, the active ingredient is combined with emulsifying and suspending agents,such as sodium CMC, methyl cellulose, pectin and gelatin. If desired, certain sweetening and/or flavoring and/or coloring agents may be added. Alternatively, the pharmaceutical compositions of this invention may be administered in the form of suppositories for rectal administration. These can be prepared by mixing the agent with a suitable nonirritating excipient which is solid at room temperature but liquid at rectal temperature and therefore will melt in the rectum to release the drug. Such materials include cocoa butter, beeswax, gelatin, glycerin and polyethylene glycols.

The pharmaceutical compositions of this invention may also be administered topically, especially when the target of treatment includes areas or organs readily accessible by topical application, including diseases of the eye or the skin. Topically-transdermal patches may also be used. For topical applications, the pharmaceutical compositions may be

formulated in a suitable ointment containing the active component suspended or dissolved in one or more carriers.

Carriers for topical administration of the compounds of this invention include, but are not limited to, mineral oil, liquid petrolatum, white petrolatum, propylene glycol, polyoxyethylene, polyoxypropylene compound, emulsifying wax, stearic acid, cetyl stearate, cetyl alcohol, lanolin, magnesium hydroxide, kaolin and water. Alternatively, the pharmaceutical compositions can be formulated in a suitable lotion or cream containing the active components suspended or dissblved in one or more pharmaceutically acceptable carriers. Suitable carriersinclude, but are not limited to, mineral oil, sorbitan monostearate, polysorbate 60, cetyl esters, wax, cetyl alcohol, 2-octyldodecanol, benzyl alcohol and water.

For ophthalmic use, the pharmaceutical compositions may be formulated as micronized suspensions in isotonic, pH adjusted sterile saline, or, preferably, as solutions in isotonic, pH adjusted sterile saline, either with our without a preservative such as benzylalkonium chloride. Alternatively, for ophthalmic uses, the pharmaceutical compositions may be formulated in an ointment such as petrolatum.

The pharmaceutical compositions of this invention may also be administered by nasal aerosol or inhalation. Such compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other conventional solubilizing or dispersing agents. The amount of either the compound or other neurotrophic, neuroprotective,

thrombolytic, and/or anti-thrombotic agents (in those compositions which comprise additional neurotrophic, neuroprotective, thrombolytic, and/or anti-thrombotic agents) that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration.

Preferably, the compositions of this invention should be formulated so that a dosage of between 0.01 - 10 mg/kg body weight/day of a compound of this invention can be administered. More preferably, the dosage is between 0.1 mg/kg body weight/day. In compositions which comprise other neurotrophic, neuroprotective, thrombolytic, and/or anti-thrombotic agents, said agents and the FGF-8 compounds act synergistically to stimulate neurite outgrowth, nerve growth, or neuronal recovery or inhibit neurodegeneration. Therefore, the amount of any other neurotrophic, neuroprotective, thrombolytic, and/or anti-thrombotic agent in such compositions will be less than that required in a monotherapy utilizing only that factor. In such compositions a dosage of between 0.01 - 10 mg/kg body weight/day of each neurotrophic, neuroprotective, thrombolytic, and/or anti-thrombotic agent can be administered. It should also be understood that a specific dosage and treatment regimen for any particular patient will depend upon a variety of factors, including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, rate of excretion, drug combination, and the judgment of the treating physician and the severity of the particular disease being treated. The amount of active ingredients will also depend upon the particular compound of this invention and the neurotrophic, neuroprotective,

thrombolytic, and/or anti-thrombotic agents used in the pharmaceutical composition.

A preferred embodiment of this aspect of the invention includes administering the FGF-8 compound and any other neurotrophic, neuroprotective, thrombolytic, and/or anti-thrombotic agent in a single dosage form when they are to be co-administered.

In another aspect of this embodiment, the pharmaceutical compositions of the present invention can be used in a method to stimulate nerve growth ex vivo. For this aspect, the compounds or compositions described above can be applied directly to the nerve cells in culture. This aspect of the invention is useful for ex vivo nerve regeneration.

According to an alternate embodiment of the present invention, the method of stimulating neurite outgrowth, enhancing nerve growth, or preventing neurodegeneration comprises the step of treating a patient or ex vivo nerve cells in culture with compositions of this invention as described above in the presence or absence of other neurotrophic, neuroprotective, thrombolytic, and/or anti-thrombotic agents.

The compositions of the present invention are also useful in bioassays measuring neurite outgrowth or neuronal migration altering activities. For example, the examples discribed herein (see in particular, Example 6 and 7) can be modified such that cell lines or cortical explants are co-cultured with cells expressing hFGF-8 polypeptides or fragments thereof. If desirable, a similar assays can be performed to identify small molecules (including hFGF-8 peptides or chemical molecules) that block hFGF-8's growth promoting effects. hFGF-8 may act as " kindling" in

disorders such as epilepsy, by contributing to sustained and increased excitability of dendrites through its neurite outgrowth promoting activity. Consequently, it would be desirable to block hFGF-8's growth promoting effects in such disorders. Further, blocking hFGF-8's migration promoting activity might be useful for treatment of various neuronal tumors including, but not limited to, glial cell tumors. The desired biological function may be obtained from polypeptides ranging in size from fragments containing a single epitope to which an antibody molecule can bind to large polypeptides which are capable of participating in the characteristic induction or programming of phenotypic changes within a cell.

As to the selection of peptides or polypeptides bearing an antigenic epitope (i.e., that contain at least a portion of a region of a polypeptide molecule to which an antibody can bind), it is well known in the art that relatively short synthetic peptides that mimic part of a polypeptide sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked polypeptide. See, for instance, J. G. Sutcliffe, et al., " Antibodies that react with preidentified sites on polypeptides," Science 219:660-666 (1983).

Antigenic epitope-bearing peptides and polypeptides of the invention are useful to raise antibodies, including monoclonal antibodies, or screen antibodies, including fragments or single chain antibodies, that bind specifically to a polypeptide of the invention. See, for instance, Wilson, *et al.*, Cell 37:767-778 (1984) at 777. Antigenic epitope-bearing peptides and polypeptides of the invention preferably contain a sequence of at least five, more preferably at least nine, and most preferably between at

least about 15 to about 30 amino acids contained within the amino acid sequence of a polypeptide of the invention.

The epitope-bearing peptides and polypeptides of the invention can be produced by any conventional means. R. A. Houghten, " General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids," Proc. Natl. Acad. Sci. USA 82:5131-5135 (1985). This " Simultaneous Multiple Peptide Synthesis (SMPS)" process is further described in U.S. Patent No. 4,631,211 to Houghten, *et al.* (1986).

As one of skill in the art will appreciate, hFGF-8 polypeptides of the present invention and the epitope-bearing fragments thereof described above can be combined with parts of the constant domain of immunoglobulins (IgG), resulting in chimeric polypeptides. These fusion proteins facilitate purification and show an increased half-life in vivo. This has been shown, e.g., for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins (EPA 394,827; Traunecker, *et al.*, Nature 331:84-86 (1988)). Fusion proteins that have a disulfide-linked dimeric structure due to the IgG part can also be more efficient in binding and neutralizing other molecules than the monomeric hFGF-8 polypeptide or polypeptide fragment alone (Fountoulakis, et al., J. Biochem. 270:3958-3964 (1995)).

Production of Antibodies

The polypeptide components or suitable fragments thereof contained in the compositions of this invention may be used in the production of antibodies. The term

" antibody" as used herein describes antibodies, fragments of antibodies (such as, but not limited, to Fab, Fab', Fab2', and Fv fragments), and modified versions thereof, as well known in the art (e.g., chimeric, humanized, recombinant, veneered, resurfaced or CDR-grafted) such antibodies are capable of binding antigens of a similar nature as the parent antibody molecule from which they are derived. The instant invention also encompasses single chain polypeptide binding molecules.

The production of antibodies, both monoclonal and polyclonal, in animals is well known in the art. See, e.g., Colligan, supra, entirely incorporated herein by reference.

Single chain antibodies and libraries thereof are yet another variety of genetically engineered antibody technology that is well known in the art. (See, e.g., R. E. Bird, *et al.*, Science 242:423-426 (1988); PCT Publication Nos. WO 88/01649, WO 90/14430, and WO 91/10737. Single chain antibody technology involves covalently joining the binding regions of heavy and light chains to generate a single polypeptide chain. The binding specificity of the intact antibody molecule is thereby reproduced on a single polypeptide chain.

Antibodies included in this invention are useful in diagnostics, therapeutics or in diagnostic/therapeutic combinations.

The polypeptides of this invention or suitable fragments thereof can be used to generate polyclonal or monoclonal antibodies, and various inter-species hybrids, or humanized antibodies, or antibody fragments, or single-chain antibodies. The techniques for producing antibodies are well known to skilled artisans. (See, e.g., Colligan supra;

Monoclonal Antibodies: Principles & Applications, Ed. J. R. Birch & E. S. Lennox, Wiley-Liss (1995).

A polypeptide used as an immunogen may be modified or administered in an adjuvant, by subcutaneous or intraperitoneal injection into, for example, a mouse or a rabbit. For the production of monoclonal antibodies, spleen cells from immunized animals are removed, fused with myeloma or other suitable known cells, and allowed to become monoclonal antibody producing hybridoma cells in the manner known to the skilled artisan. Hybridomas that secrete a desired antibody molecule can be screened by a variety of well known methods, for example ELISA assay, Western blot analysis, or radioimmunoassay (Lutz, *et al.* Exp. Cell Res. 175:109-124 (1988); Monoclonal Antibodies: Principles & Applications, Ed. J. R. Birch & E. S. Lennox, Wiley-Liss (1995); Colligan, supra).

For some applications labeled antibodies are desirable. Procedures for labeling antibody molecules are widely known, including for example, the use of radioisotopes, affinity labels, such as biotin or avidin, enzymatic labels, for example horseradish peroxidase, and fluorescent labels, such as FITC or rhodamine (See, e.g., Colligan, supra).

Labeled antibodies are useful for a variety of diagnostic applications. In one embodiment the present invention relates to the use of labeled antibodies to detect the presence of an hFGF-8 polypeptide. Alternatively, the antibodies could be used in a screen to identify potential modulators of an hFGF-8 polypeptide. For example, in a competitive displacement assay, the antibody or compound to be tested is labeled by any suitable method. Competitive displacement of an antibody from an antibody-antigen complex by a test compound such that a test compound-antigen complex

is formed provides a method for identifying compounds that bind HPLFP.

Transgenics and Chimeric Non-Human Mammals

The present invention is also directed to a transgenic non-human eukaryotic animal (preferably a rodent, such as a mouse) the germ cells and somatic cells of which contain nucleic acid genomic DNA according to the present invention which codes for at least one hFGF-8 polypeptide. At least one hFGF-8 nucleic acid can be introduced into the animal to be made transgenic, or an ancestor of the animal, at an embryonic stage, preferably the 1-1000 cell or oocyte, stage, and preferably not later than about the 64-cell stage. The term " transgene," as used herein, means a gene which is incorporated into the genome of the animal and is expressed in the animal, resulting in the presence of at least one hFGF-8 polypeptide in the transgenic animal.

There are several means by which such an hFGF-8 nucleic acid can be introduced into a cell or genome of the animal embryo so as to be chromosomally incorporated and expressed according to known methods.

Chimeric non-human mammals in which fewer than all of the somatic and germ cells contain the an hFGF-8 polypeptide nucleic acid of the present invention, such as animals produced when fewer than all of the cells of the morula are transfected in the process of producing the transgenic animal, are also intended to be within the scope of the present invention.

Chimeric non-human mammals having human cells or tissue engrafted therein are also encompassed by the present invention, which may be used for testing expression of at least one hFGF-8 polypeptide in human tissue and/or for

testing the effectiveness of therapeutic and/or diagnostic agents associated with delivery vectors which preferentially bind to an hFGF-8 polypeptide of the present invention. Methods for providing chimeric non-human mammals are provided, e.g., in U.S. Serial Nos. 07/508,225, 07/518,748, 07/529,217, 07/562,746, 07/596,518, 07/574,748, 07/575,962, 07/207,273, 07/241,590 and 07/137,173, which are entirely incorporated herein by reference, for their description of how to engraft human cells or tissue into non-human mammals.

The techniques described in Leder, U.S. Patent No. 4,736,866 (hereby entirely incorporated by reference) for producing transgenic non-human mammals may be used for the production of a transgenic non-human mammal of the present invention. The various techniques described in U.S. patent Nos. 5,454,807, 5,073,490, 5,347,075 and 4,736,866, the entire contents of which are hereby incorporated by reference, may also be used.

Animals carrying at least one hFGF-8 polypeptide and/or nucleic acid can be used to test compounds or other treatment modalities which may prevent, suppress or cure a pathology relating to at least one hFGF-8 polypeptide or hFGF-8 nucleic acid. Such transgenic animals will also serve as a model for testing of diagnostic methods for the same diseases. Transgenic animals according to the present invention can also be used as a source of cells for cell culture.

Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

Example 1: Expression and Purification of an hFGF-8 Polypeptide in E. coli

The bacterial expression vector pQE60 is used for bacterial expression in this example. (QIAGEN, Inc., Chatsworth, CA). pQE60 encodes ampicillin antibiotic resistance (" Ampr") and contains a bacterial origin of replication (" ori"), an IPTG inducible promoter, a ribosome binding site (" RBS"), six codons encoding histidine residues that allow affinity purification using nickel-nitrilo-tri-acetic acid (" Ni-NTA") affinity resin sold by QIAGEN, Inc., and suitable single restriction enzyme cleavage sites. These elements are arranged such that a DNA fragment encoding a polypeptide can be inserted in such a way as to produce that polypeptide with the six His residues (i.e., a " 6 X His tag") covalently linked to the carboxyl terminus of that polypeptide. However, a polypeptide coding sequence can optionally be inserted such that translation of the six His codons is prevented and, therefore, a polypeptide is produced with no 6 X His tag.

The nucleic acid sequence encoding the desired portion of an hFGF-8 polypeptide lacking the hydrophobic leader sequence is amplified from the deposited cDNA clone using PCR oligonucleotide primers (based on the sequences presented, e.g., as presented in at least one of SEQ ID NOS:1, 2, 3, and 8), which anneal to the amino terminal encoding DNA sequences of the desired portion of an hFGF-8 polypeptide and to sequences in the deposited construct 3' to the cDNA coding sequence. Additional nucleotides containing restriction sites to facilitate cloning in the pQE60 vector are added to the 5' and 3' sequences, respectively.

For cloning an hFGF-8 polypeptide, the 5' and 3' primers have nucleotides corresponding or complementary to a portion of the coding sequence of an hFGF-8, e.g., as presented in at least one of SEQ ID NOS:1, 2, 3, and 8, according to known method steps. One of ordinary skill in the art would appreciate, of course, that the point in a polypeptide coding sequence where the 5' primer begins can be varied to amplify a desired portion of the complete polypeptide shorter or longer than the mature form.

The amplified hFGF-8 nucleic acid fragments and the vector pQE60 are digested with appropriate restriction enzymes and the digested DNAs are then ligated together. Insertion of the hFGF-8 DNA into the restricted pQE60 vector places an hFGF-8 polypeptide coding region including its associated stop codon downstream from the IPTG-inducible promoter and in-frame with an initiating AUG codon. The associated stop codon prevents translation of the six histidine codons downstream of the insertion point.

The ligation mixture is transformed into competent E. coli cells using *standard procedures such as those described in Sambrook, et* al., 1989; Ausubel, 1987-1998. E. coli strain M15/rep4, containing multiple copies of the plasmid pREP4, which expresses the lac repressor and confers kanamycin resistance (" Kanr"), is used in carrying out the illustrative example described herein. This strain, which is only one of many that are suitable for expressing hFGF-8 polypeptide, is available commercially from QIAGEN, Inc. Transformants are identified by their ability to grow on LB plates in the presence of ampicillin and kanamycin. Plasmid DNA is isolated from resistant colonies and the identity of the cloned DNA confirmed by restriction analysis, PCR and DNA sequencing.

Clones containing the desired constructs are grown overnight (" O/N") in liquid culture in LB media supplemented with both ampicillin (100 µg/ml) and kanamycin (25 µg/ml). The O/N culture is used to inoculate a large culture, at a dilution of approximately 1:25 to 1:250. The cells are grown to an optical density at 600 nm (" OD600") of between 0.4 and 0.6. Isopropyl-b-D-thiogalactopyranoside (" IPTG") is then added to a final concentration of 1 mM to induce transcription from the lac repressor sensitive promoter, by inactivating the lacI repressor. Cells subsequently are incubated further for 3 to 4 hours. Cells then are harvested by centrifugation.

The cells are then stirred for 3-4 hours at 4°C in 6M guanidine-HCl, pH8. The cell debris is removed by centrifugation, and the supernatant containing the hFGF-8 is dialyzed against 50 mM Na-acetate buffer pH6, supplemented with 200 mM NaCl. Alternatively, a polypeptide can be successfully refolded by dialyzing it against 500 mM NaCl, 20% glycerol, 25 mM Tris/HCl pH7.4, containing protease inhibitors.

If insoluble protein is generated, the protein is made soluble according to known method steps. After renaturation the polypeptide is purified by ion exchange, hydrophobic interaction and size exclusion chromatography. Alternatively, an affinity chromatography step such as an antibody column is used to obtain pure hFGF-8 polypeptide. The purified polypeptide is stored at 4°C or frozen at -40°C to -120°C.

Example 2: Cloning and Expression of an hFGF-8 Polypeptide in a Baculovirus Expression System

In this illustrative example, the plasmid shuttle vector pA2 GP is used to insert the cloned DNA encoding the mature polypeptide into a baculovirus to express an hFGF-8 polypeptide, using a baculovirus leader and standard methods as described in Summers, et al., A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures, Texas Agricultural Experimental Station Bulletin No. 1555 (1987). This expression vector contains the strong polyhedrin promoter of the Autographa californica nuclear polyhedrosis virus (AcMNPV) followed by the secretory signal peptide (leader) of the baculovirus gp67 polypeptide and convenient restriction sites such as BamHI, Xba I and Asp718. The polyadenylation site of the simian virus 40 (" SV40") is used for efficient polyadenylation. For easy selection of recombinant virus, the plasmid contains the beta-galactosidase gene from E. coli under control of a weak Drosophila promoter in the same orientation, followed by the polyadenylation signal of the polyhedrin gene. The inserted genes are flanked on both sides by viral sequences for cell-mediated homologous recombination with wild-type viral DNA to generate viable virus that expresses the cloned polynucleotide.

Other baculovirus vectors are used in place of the vector above, such as pAc373, pVL941 and pAcIM1, as one skilled in the art would readily appreciate, as long as the construct provides appropriately located signals for transcription, translation, secretion and the like, including a signal peptide and an in-frame AUG as required. Such vectors are described, for instance, in Luckow, *et al.*, Virology 170:31-39.

The cDNA sequence encoding the mature hFGF-8 polypeptide in the deposited or other clone, lacking the AUG initiation codon and the naturally associated nucleotide binding site, is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene. Non-limiting examples include 5' and 3' primers having nucleotides corresponding or complementary to a portion of the coding sequence of an hFGF-8 polypeptide, e.g., as presented in at least one of SEQ ID NOS:1, 2, 3, and 8, according to known method steps.

The amplified fragment is isolated from a 1% agarose gel using a commercially available kit (e.g., " Geneclean," BIO 101 Inc., La Jolla, CA). The fragment then is then digested with the appropriate restriction enzyme and again is purified on a 1% agarose gel. This fragment is designated herein " F1" .

The plasmid is digested with the corresponding restriction enzymes and optionally, can be dephosphorylated using calf intestinal phosphatase, using routine procedures known in the art. The DNA is then isolated from a 1% agarose gel using a commercially available kit (" Geneclean" BIO 101 Inc., La Jolla, CA). This vector DNA is designated herein " V1" .

Fragment F1 and the dephosphorylated plasmid V1 are ligated together with T4 DNA ligase. E. coli HB101 or other suitable E. coli hosts such as XL-1 Blue (Stratagene Cloning Systems, La Jolla, CA) cells are transformed with the ligation mixture and spread on culture plates. Bacteria are identified that contain the plasmid with the human hFGF-8 gene using the PCR method, in which one of the primers that is used to amplify the gene and the second primer is from well within the vector so that only those bacterial colonies

containing the hFGF-8 gene fragment will show amplification of the DNA. The sequence of the cloned fragment is confirmed by DNA sequencing. This plasmid is designated herein pBac hFGF-8.

Five µg of the plasmid pBachFGF-8 is co-transfected with 1.0 µg of a commercially available linearized baculovirus DNA (" BaculoGold™ baculovirus DNA" , Pharmingen, San Diego, CA), using the lipofection method described by Felgner, *et al.*, Proc. Natl. Acad. Sci. USA 84:7413-7417 (1987). 1 µg of BaculoGold™ virus DNA and 5 µg of the plasmid pBac hFGF-8 are mixed in a sterile well of a microtiter plate containing 50 µl of serum-free Grace's medium (Life Technologies, Inc., Rockville, MD). Afterwards, 10 µl Lipofectin plus 90 µl Grace's medium are added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture is added drop-wise to Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate is rocked back and forth to mix the newly added solution. The plate is then incubated for 5 hours at 27°C. After 5 hours the transfection solution is removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum is added. The plate is put back into an incubator and cultivation is continued at 27°C for four days.

After four days the supernatant is collected and a plaque assay is performed, according to known methods. An agarose gel with " Blue Gal" (Life Technologies, Inc., Rockville, MD) is used to allow easy identification and isolation of gal-expressing clones, which produce blue-stained plaques. (A detailed description of a " plaque assay" of this type can also be found in the user's guide

for insect cell culture and baculovirology distributed by Life Technologies, Inc., Rockville, MD, page 9-10). After appropriate incubation, blue stained plaques are picked with a micropipettor tip (e.g., Eppendorf). The agar containing the recombinant viruses is then resuspended in a microcentrifuge tube containing 200 µl of Grace's medium and the suspension containing the recombinant baculovirus is used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes are harvested and then they are stored at 4°C. The recombinant virus is called V-hFGF-8.

To verify the expression of the hFGF-8 gene, Sf9 cells are grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells are infected with the recombinant baculovirus V-hFGF-8 at a multiplicity of infection ("MOI") of about 2. Six hours later the medium is removed and is replaced with SF900 II medium minus methionine and cysteine (available, e.g., from Life Technologies, Inc., Rockville, MD). If radiolabeled polypeptides are desired, 42 hours later, 5 mCi of 35S-methionine and 5 mCi 35S-cysteine (available from Amersham) are added. The cells are further incubated for 16 hours and then they are harvested by centrifugation. The polypeptides in the supernatant as well as the intracellular polypeptides are analyzed by SDS-PAGE followed by autoradiography (if radiolabeled). Microsequencing of the amino acid sequence of the amino terminus of purified polypeptide can be used to determine the amino terminal sequence of the mature polypeptide and thus the cleavage point and length of the secretory signal peptide.

Example 3: Cloning and Expression of hFGF-8 in Mammalian Cells

A typical mammalian expression vector contains at least one promoter element, which mediates the initiation of transcription of mRNA, the polypeptide coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription can be achieved with the early and late promoters from SV40, the long terminal repeats (LTRS) from Retroviruses, e.g., RSV, HTLVI, HIVI and the early promoter of the cytomegalovirus (CMV). However, cellular elements can also be used (e.g., the human actin promoter). Suitable expression vectors for use in practicing the present invention include, for example, vectors such as pIRES1neo, pRetro-Off, pRetro-On, PLXSN, or pLNCX (Clonetech Labs, Palo Alto, CA), pcDNA3.1 (+/-), pcDNA/Zeo (+/-) or pcDNA3.1/Hygro (+/-) (Invitrogen), PSVL and PMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146) and pBC12MI (ATCC 67109). Mammalian host cells that could be used include human Hela 293, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 7 and CV 1, quail QC1-3 cells, mouse L cells and Chinese hamster ovary (CHO) cells.

Alternatively, the gene can be expressed in stable cell lines that contain the gene integrated into a chromosome. The co-transfection with a selectable marker such as dhfr, gpt, neomycin, or hygromycin allows the identification and isolation of the transfected cells.

The transfected gene can also be amplified to express large amounts of the encoded polypeptide. The DHFR

(dihydrofolate reductase) marker is useful to develop cell lines that carry several hundred or even several thousand copies of the gene of interest. Another useful selection marker is the enzyme glutamine synthase (GS) (Murphy, *et al.*, Biochem. J. 227:277-279 (1991); Bebbington, *et al.*, Bio/Technology 10:169-175 (1992)). Using these markers, the mammalian cells are grown in selective medium and the cells with the highest resistance are selected. These cell lines contain the amplified gene(s) integrated into a chromosome. Chinese hamster ovary (CHO) and NSO cells are often used for the production of polypeptides.

The expression vectors pC1 and pC4 contain the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen, *et al.*, Molec. Cell. Biol. 5:438-447 (1985)) plus a fragment of the CMV-enhancer (Boshart, *et al.*, Cell 41:521-530 (1985)). Multiple cloning sites, e.g., with the restriction enzyme cleavage sites BamHI, XbaI and Asp718, facilitate the cloning of the gene of interest. The vectors contain in addition the 3' intron, the polyadenylation and termination signal of the rat preproinsulin gene.

Example 3(a): Cloning and Expression in COS Cells

The expression plasmid, phFGF-8 HA, is made by cloning a cDNA encoding hFGF-8 into the expression vector pcDNAI/Amp or pcDNAIII (which can be obtained from Invitrogen, Inc.).

The expression vector pcDNAI/amp contains: (1) an E. coli origin of replication effective for propagation in E. coli and other prokaryotic cells; (2) an ampicillin resistance gene for selection of plasmid-containing prokaryotic cells; (3) an SV40 origin of replication for propagation in eucaryotic cells; (4) a CMV promoter, a polylinker, an SV40 intron; (5) several codons encoding a

hemagglutinin fragment (i.e., an " HA" tag to facilitate purification) or HIS tag (see, e.g, Ausubel, supra) followed by a termination codon and polyadenylation signal arranged so that a cDNA can be conveniently placed under expression control of the CMV promoter and operably linked to the SV40 intron and the polyadenylation signal by means of restriction sites in the polylinker. The HA tag corresponds to an epitope derived from the influenza hemagglutinin polypeptide described by Wilson, *et al.*, Cell 37:767-778 (1984). The fusion of the HA tag to the target polypeptide allows easy detection and recovery of the recombinant polypeptide with an antibody that recognizes the HA epitope. pcDNAIII contains, in addition, the selectable neomycin marker.

A DNA fragment encoding the hFGF-8 is cloned into the polylinker region of the vector so that recombinant polypeptide expression is directed by the CMV promoter. The plasmid construction strategy is as follows. The hFGF-8 cDNA of the deposited clone is amplified using primers that contain convenient restriction sites, much as described above for construction of vectors for expression of hFGF-8 in E. coli. Non-limiting examples of suitable primers include those based on the coding sequences presented in at least one of SEQ ID NOS:1, 2, 3, and 8, as they encode hFGF-8 polypeptides as described herein.

The PCR amplified DNA fragment and the vector, pcDNAI/Amp, are digested with suitable restriction enzyme(s) and then ligated. The ligation mixture is transformed into E. coli strain SURE (available from Stratagene Cloning Systems, 11099 North Torrey Pines Road, La Jolla, CA 92037), and the transformed culture is plated on ampicillin media plates which then are incubated to allow growth of

ampicillin resistant colonies. Plasmid DNA is isolated from resistant colonies and examined by restriction analysis or other means for the presence of the hFGF-8-encoding fragment.

For expression of recombinant hFGF-8, COS cells are transfected with an expression vector, as described above, using DEAE-DEXTRAN, as described, for instance, in Sambrook, et al., Molecular Cloning: a Laboratory Manual, Cold Spring Laboratory Press, Cold Spring Harbor, New York (1989). Cells are incubated under conditions for expression of hFGF-8 by the vector.

Expression of the hFGF-8-HA fusion polypeptide is detected by radiolabeling and immunoprecipitation, using methods described in, for example Harlow, et al., Antibodies: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1988). To this end, two days after transfection, the cells are labeled by incubation in media containing 35S-cysteine for 8 hours. The cells and the media are collected, and the cells are washed and lysed with detergent-containing RIPA buffer: 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% DOC, 50 mM TRIS, pH 7.5, as described by Wilson, et al. cited above. Proteins are precipitated from the cell lysate and from the culture media using an HA-specific monoclonal antibody. The precipitated polypeptides then are analyzed by SDS-PAGE and autoradiography. An expression product of the expected size is seen in the cell lysate, which is not seen in negative controls.

Example 3(b): Cloning and Expression in CHO Cells

The vector pC4 is used for the expression of hFGF-8 polypeptide. Plasmid pC4 is a derivative of the plasmid

pSV2-dhfr (ATCC Accession No. 37146). The plasmid contains the mouse DHFR gene under control of the SV40 early promoter. Chinese hamster ovary- or other cells lacking dihydrofolate activity that are transfected with these plasmids can be selected by growing the cells in a selective medium (alpha minus MEM, Life Technologies) supplemented with the chemotherapeutic agent methotrexate. The amplification of the DHFR genes in cells resistant to methotrexate (MTX) has been well documented (see, e.g., F. W. Alt, et al., J. Biol. Chem. 253:1357-1370 (1978); J. L. Hamlin and C. Ma, Biochem. et Biophys. Acta 1097:107-143 (1990); and M. J. Page and M. A. Sydenham, Biotechnology 9:64-68 (1991)). Cells grown in increasing concentrations of MTX develop resistance to the drug by overproducing the target enzyme, DHFR, as a result of amplification of the DHFR gene. If a second gene is linked to the DHFR gene, it is usually co-amplified and over-expressed. It is known in the art that this approach can be used to develop cell lines carrying more than 1,000 copies of the amplified gene(s). Subsequently, when the methotrexate is withdrawn, cell lines are obtained which contain the amplified gene integrated into one or more chromosome(s) of the host cell.

Plasmid pC4 contains for expressing the gene of interest the strong promoter of the long terminal repeat (LTR) of the Rous Sarcoma Virus (Cullen, *et al.*, Molec. Cell. Biol. 5:438-447 (1985)) plus a fragment isolated from the enhancer of the immediate early gene of human cytomegalovirus (CMV) (Boshart, et al., Cell 41:521-530 (1985)). Downstream of the promoter are BamHI, XbaI, and Asp718 restriction enzyme cleavage sites that allow integration of the genes. Behind these cloning sites the plasmid contains the 3' intron and polyadenylation site of

the rat preproinsulin gene. Other high efficiency promoters can also be used for the expression, e.g., the human b-actin promoter, the SV40 early or late promoters or the long terminal repeats from other retroviruses, e.g., HIV and HTLVI. Clontech's Tet-Off and Tet-On gene expression systems and similar systems can be used to express the hFGF-8 in a regulated way in mammalian cells (M. Gossen, and H. Bujard, Proc. Natl. Acad. Sci. USA 89: 5547-5551 (1992)). For the polyadenylation of the mRNA other signals, e.g., from the human growth hormone or globin genes can be used as well. Stable cell lines carrying a gene of interest integrated into the chromosomes can also be selected upon co-transfection with a selectable marker such as gpt, G418 or hygromycin. It is advantageous to use more than one selectable marker in the beginning, e.g., G418 plus methotrexate.

The plasmid pC4 is digested with restriction enzymes and then dephosphorylated using calf intestinal phosphatase by procedures known in the art. The vector is then isolated from a 1% agarose gel.

The DNA sequence encoding the complete hFGF-8 polypeptide is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene. Non-limiting examples include 5' and 3' primers having nucleotides corresponding or complementary to a portion of the coding sequence of an hFGF-8, e.g., as presented in at least one of SEQ ID NOS:1, 2, 3, and 8, according to known method steps.

The amplified fragment is digested with suitable endonucleases and then purified again on a 1% agarose gel. The isolated fragment and the dephosphorylated vector are then ligated with T4 DNA ligase. E. coli HB101 or XL-1 Blue

cells are then transformed and bacteria are identified that contain the fragment inserted into plasmid pC4 using, for instance, restriction enzyme analysis.

Chinese hamster ovary (CHO) cells lacking an active DHFR gene are used for transfection. 5 µg of the expression plasmid pC4 is cotransfected with 0.5 µg of the plasmid pSV2-neo using lipofectin. The plasmid pSV2neo contains a dominant selectable marker, the neo gene from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The cells are seeded in alpha minus MEM supplemented with 400 µg/ml G418. After 2 days, the cells are trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) in alpha minus MEM supplemented with 10, 25, or 50 ng/ml of methotrexate plus 400 µg/ml G418. After about 10-14 days single clones are trypsinized and then seeded in 6-well petri dishes or 10 ml flasks using different concentrations of methotrexate (50 nM, 100 nM, 200 nM, 400 nM, 800 nM). Clones growing at the highest concentrations of methotrexate are then transferred to new 6-well plates containing even higher concentrations of methotrexate (1 µM, 2 µM, 5 µM, 10 µM, 20 µM). The same procedure is repeated until clones are obtained which grow at a concentration of 100 - 200 µM. Expression of the desired gene product is analyzed, for instance, by SDS-PAGE and Western blot or by reverse phase HPLC analysis.

Example 4: Tissue Distribution of hFGF-8 mRNA Expression

Northern blot analysis is carried out to examine hFGF-8 gene expression in human tissues, using methods described by, among others, Sambrook, et al., cited above. A cDNA probe containing the entire nucleotide sequence of an hFGF-8 polypeptide (SEQ ID NO:1) is labeled with ^{32}P using the

Rediprime™ DNA labeling system (Amersham Life Science), according to the manufacturer's instructions. After labeling, the probe is purified using a CHROMA SPIN-100™ column (Clontech Laboratories, Inc.), according to the manufacturer's protocol number PT1200-1. The purified and labeled probe is used to examine various human tissues for hFGF-8 mRNA.

Multiple Tissue Northern (MTN) blots containing various human tissues (H) or human immune system tissues (IM) are obtained from Clontech and are examined with the labeled probe using ExpressHyb hybridization solution (Clontech) according to manufacturer's protocol number PT1190-1. Following hybridization and washing, the blots are mounted and exposed to film at -70°C overnight, and films developed according to standard procedures. The results show hFGF-8 polypeptides to be selectively expressed in at least one of brain and other tissues.

Example 5: Directed Mutagenesis of hFGF-8 Polypeptides to Provide DNA Encoding Specified Substitutions, Insertions or Deletions of SEQ ID NO:2 Using the Polymerase Chain Reaction

The polymerase chain reaction (PCR) can be used for the enzymatic amplification and direct sequencing of small quantities of nucleic acids (see, e.g., Ausubel, supra, section 15) to provide specified substitutions, insertions or deletions in DNA encoding an hFGF-8 polypeptide of the present inventions, e.g., SEQ ID NO:2 (e.g., SEQ ID NO:1, or any sequence described herein), as presented herein, to provide an hFGF-8 polypeptide sequence of interest including at least one substitution, insertion or deletion of SEQ ID NOS:4, 5, 6, and 7. This technology can be used as a quick

and efficient method for introducing any desired sequence change into the DNA of interest.

Unit 8.5 of Ausubel, supra, contains two basic protocols for introducing base changes into specific DNA sequences. Basic Protocol 1, as presented in the first section 8.5 of Ausubel, supra (entirely incorporated herein by reference), describes the incorporation of a restriction site and Basic Protocol 2, as presented below and in the second section of Unit 8.5 of Ausubel, supra, details the generation of specific point mutations (all of the following references in this example are to sections of Ausubel, et al., eds., Current Protocols in Molecular Biology, Wiley Interscience, New York (1987-1999)). An alternate protocol describes generating point mutations by sequential PCR steps. Although the general procedure is the same in all three protocols, there are differences in the design of the synthetic oligonucleotide primers and in the subsequent cloning and analyses of the amplified fragments.

The PCR procedure described here can rapidly, efficiently, and/or reproducibly introduce any desired change into a DNA fragment. It is similar to the oligonucleotide-directed mutagenesis method described in UNIT 8.1, but does not require the preparation of a uracil-substituted DNA template.

The main disadvantage of PCR-generated mutagenesis is related to the fidelity of the Taq DNA polymerase. The mutation frequency for Taq DNA polymerase was initially estimated to be as high as 1/5000 per cycle (Saiki et al., 1988). This means that the entire amplified fragment must be sequenced to be sure that there are no Taq-derived mutations. To reduce the amount of sequencing required, it is best to introduce the mutation by amplifying as small a

fragment as possible. With rapid and reproducible methods of double-stranded DNA sequencing (UNIT 7.4), the entire amplified fragment can usually be sequenced from a single primer. If the fragment is somewhat longer, it is best to subclone the fragment into an M13-derived vector, so that both forward and reverse primers can be used to sequence the amplified fragment.

If there are no convenient restriction sites flanking the fragment of interest, the utility of this method is somewhat reduced. Many researchers prefer the mutagenesis procedure in UNIT 8.1 to avoid excessive sequencing.

A full discussion of critical parameters for PCR amplification can be found in UNIT 15.1.

Anticipated Results

Each of the procedures presented here has a 100% efficiency rate. All or substantially all of the cloned, amplified fragments will contain the mutation corresponding to the synthesized oligonucleotide.

Literature Cited

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487-491.

BASIC PROTOCOL (2): INTRODUCTION OF POINT MUTATIONS BY PCR

In this protocol, synthetic oligonucleotides are designed to incorporate a point mutation at one end of an amplified fragment. Following PCR, the amplified fragments are made blunt-ended by treatment with Klenow fragment. These fragments are then ligated and subcloned into a vector

to facilitate sequence analysis. This procedure is summarized in Figure 8.5.2 of Ausubel, supra.

Materials

DNA sample to be mutagenized

Klenow fragment of E. coli DNA polymerase I (UNIT 3.5 of Ausubel, supra)

Appropriate restriction endonuclease (Table 8.5.1)

Additional reagents and equipment for synthesis and purification of oligonucleotides (UNITS 2.11 & 2.12), phosphorylation of oligonucleotides (UNIT 3.10), electrophoresis of DNA on nondenaturing agarose and low gelling/melting agarose gels (UNITS 2.5A & 2.6), restriction endonuclease digestion (UNIT 3.1), ligation of DNA fragments (UNIT 3.16), transformation of E. coli (UNIT 1.8), plasmid DNA miniprep (UNIT 1.6), and DNA sequence analysis (UNIT 7.4)

Prepare the template DNA and oligonucleotide primers

Prepare template DNA (see Basic Protocol 1, steps 1 and 2). Synthesize (UNIT 2.11) and purify (UNIT 2.12) the oligonucleotide primers (primers 3 and 4 in Fig. 8.5.2B). The oligonucleotide primers must be homologous to the template DNA for more than 15 bases. No four-base " clamp" sequence is added to these primers. The primer sequences are based on a DNA encoding the hFGF-8 polypeptide sequence of interest including at least one substitution, insertion or deletion of SEQ ID NOS:4, 5, 6, and 7. Phosphorylate the 5′ end of the oligonucleotides (UNIT 3.10). This step is necessary because the 5′ end of the oligonucleotide will be used directly in cloning.

Amplify DNA and prepare blunt-end fragments

Amplify the template DNA (see Basic Protocol 1, steps 5 and 6). After the final extension step, add 5 U Klenow fragment to the reaction mix and incubate 15 min at 30°C. During PCR, the Taq polymerase adds an extra nontemplated nucleotide to the 3′ end of the fragment. The 3′-5′ exonuclease activity of the Klenow fragment is required to make the ends flush and suitable for blunt-end cloning (UNIT 3.5). Analyze and process the reaction mix (see Basic Protocol 1, steps 7 and 8). Digest half the amplified fragments with the restriction endonucleases for the flanking sequences (UNIT 3.1). Purify digested fragments on a low gelling/melting agarose gel (UNIT 2.6).

Subclone the two amplified fragments into an appropriately digested vector by blunt-end ligation (UNIT 3.16). Transform recombinant plasmid into E. coli (UNIT 1.8). Prepare DNA by plasmid miniprep (UNIT 1.6). Analyze the amplified fragment portion of the plasmid DNA by DNA sequencing to confirm the point mutation (UNIT 7.4). This is critical because the Taq DNA polymerase can introduce additional mutations into the fragment (see Critical Parameters).

ALTERNATE PROTOCOL: INTRODUCTION OF A POINT MUTATION BY SEQUENTIAL PCR STEPS

In this procedure, the two fragments encompassing the mutation are annealed with each other and extended by mutually primed synthesis; this fragment is then amplified by a second PCR step, thereby avoiding the blunt-end ligation required in Basic Protocol 2. This strategy is outlined in Figure 8.5.3. For materials, see Basic Protocols 1 and 2 of Ausubel, supra.

Prepare template DNA (see Basic Protocol 1, steps 1 and 2). Synthesize (UNIT 2.11) and purify (UNIT 2.12) the oligonucleotide primers (primers 5 and 6 in Fig. 8.5.3B) to generate an hFGF-8 polypeptide sequence of interest including at least one substitution, insertion or deletion of SEQ ID NOS:4, 5, 6, and 7. The oligonucleotides must be homologous to the template for 15 to 20 bases and must overlap with one another by at least 10 bases. The 5' end does not have a " clamp" sequence.

Amplify the template DNA and generate blunt-end fragments (see Basic Protocol 2, steps 4 and 5). Purify the fragments by nondenaturing agarose gel electrophoresis (UNIT 2.5A). Resuspend in TE buffer at 1 ng/µl.

Carry out second PCR amplification. Combine the following in a 500-µl microcentrifuge tube:

10 µl (10 ng) each amplified fragment

1 µl (500 ng) each flanking sequence primer (each 1 µM final)

10 µl 10x amplification buffer

10 µl 2 mM 4dNTP mix

H_2O to 99.5 µl

0.5 µl Taq DNA polymerase (5 U/µl).

Overlay with 100 µl mineral oil. Carry out PCR for 20 to 25 cycles, using the conditions for introduction of restriction endonuclease sites by PCR (see Basic Protocol 1, step 6). Analyze and process the reaction mix (see Basic Protocol 1, Ausubel, supra, steps 7 and 8).

Digest the DNA fragment with the appropriate restriction endonuclease for the flanking sites (UNIT 3.1). Purify the digested fragment on a low gelling/melting agarose gel (UNIT 2.6). Subclone into an appropriately

digested vector. Transform recombinant plasmid into E. coli (UNIT 1.8). Prepare DNA by plasmid miniprep (UNIT 1.6). Analyze the amplified fragment portion of the plasmid DNA by DNA sequencing (UNIT 7.4) to confirm the point mutation. This is critical because the Taq DNA polymerase can introduce additional mutations into the fragment (see Critical Parameters).

Example 6: FGF-8 Induced Neurite Outgrowth in PC12 Cells

In order to directly determine the neurotrophic activity of FGF-8 compounds or compositions described in this invention, a neurite outgrowth assay can be performed with cells or cell lines derived from neuronal tissue essentially as described by Lyons et al., (1994). Briefly, PC12 cells are maintained at 37'C and 5% CO_z in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated horse serum, 5% heat inactivated fetal bovine serum (FBS), and 1% glutamate. The cells are then plated at 105 per well in 96 well plates coated with 5 m/cm2 rat tail collagen and allowed to attach overnight. The medium is then replaced with DMEM, 2% heat-inactivated horse serum, 1% glutamate, 1-5 ng/ml of NGF (Sigma) and varying concentrations of the FGF-8 compound (0.001 nM - 100 μM). The background control culture is administered with 105 ng/ml of NGF alone without compound. Positive control culture's are administered with high concentration of NGF (50 ng/ml). The cells are then incubated at 37°C at 5% CO2 for 72 hours, fixed with 3% formaldehyde and nerve outgrowth can be determined visually and scored on a scale of 0 to 4. The assay should be done at least in triplicate for each concentration tested.

Example 7: FGF-8 Induced Neurite Outgrowth in Human Neuronal SHSY-5Y Cells

Stimulation of neurite outgrowth by FGF-8 can also be demonstrated using the human neuronal SHSY-5Y cell line (ATCC). SHSY-5 Y cells are cultured and maintained in a 1:1 mixture of Eagle's Minimum Essential Medium and Ham's F-12 medium containing 10% feral bovine serum. For neurite outgrowth, cell are plated in chamber coverslips coated with Metrigel (Collaborative Research Inc.) at a density of 20,000 cells per chamber. FGF-8b (R&D Systems, Inc.) was added at various concentrations (0.001 nM - 100 μM). After 48 to 72 hours, cells were fixed with 5% Zn-formaline and stained with H&E stain. Neurite outgrowth was evaluated by phase contrast microscopy and can be scored as in Example 6. Neurite outgrowth can also be determined by measuring the expression of any known molecular marker of new axonal growth, including but not limited to the neuronal growth-associated protein 43 (GAP43) by any means know in the art. GAP-43 is a phosphoprotein component of the neuronal membrane and growth cone that is selectively upregulated during new axonal growth in both the peripheral and central nervous systems. GAP-43 has previously been used as a reliable marker of new axonal growth during brain development, and following brain injury or ischemia (Kawamata et al., (1997(a)). Such axonal sprouting is likely to be accompanied by new dendritic sprouting and synapse formation in the intact uninjured brain. This in turn leads to enhanced recovery from neuronal damage as a result of disease of physical trauma. Immunostaining with axonal specific protein GAP43 followed by fluorescence microscopy according to known procedures shows that FGF-8

compounds resulted in a significant increase in neurite outgrowth over background control cultures (see Figure 2).

We claim:

1. A composition comprising at least one isolated polynucleotide, or complement thereof, encoding at least 90-100% of the contiguous amino acids of an hFGF-8 polypeptide selected from at least one of SEQ ID NOS:4, 5, 6, and 7 wherein said composition has neurotrophic activity.

2. A composition according to claim 1 wherein said polynucleotide, or complement thereof, further comprises at least one mutation that results in at least one amino acid substitution, insertion, or deletion of said hFGF-8 polypeptide.

3. A composition comprising a recombinant vector comprising at least one polynucleotide, or complement thereof, encoding at least 90-100% of the contiguous amino acids of a protein selected from at least one of SEQ ID NOS:4, 5, 6, and 7 wherein said composition has neurotrophic activity.

4. The composition according to claim 3 wherein said polynucleotide, or complement thereof, further comprises at least one mutation that results in at least one amino acid substitution, insertion, or deletion of said hFGF-8 polypeptide.

5. The composition according to claims 3-4 which further comprises a host cell which harbors said recombinant vector.

6. A composition comprising an isolated hFGF-8 polypeptide comprising at least 90-100% of the contiguous amino acids of at least one amino acid sequence of SEQ ID NOS:4. 5, 6, and 7 wherein said composition has neurotrophic activity.

7. The composition according to claim 9 wherein said hFGF-8 polypeptide further comprises at least one mutation corresponding to at least one substitution, insertion or deletion of SEQ ID NOS:4, 5, 6, and 7, respectively.

8. The composition according to any of claims 1-7 which further comprises a carrier or diluent.

9. The composition according to any of claims 1-8, further comprising at least one other neurotrophic, neuroprotective, thrombolytic, and/or anti-thrombotic agent wherein said agent is selected from the group consisting of nerve growth factor (NGF), insulin growth factor (IGF) and active truncated derivatives thereof, acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF) , platelet-derived growth factors (PDGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factors (CNTF), glial cell-derived neurotrophic factor 4GDNF), neurotrophin-3 (NT-31 and neurotrophin 4/5 (NT4/5).

10. A method for enhancing neuronal growth, regeneration, or survival in a patient or in an ex vivo nerve cell comprising the step of administering to said patient or said nerve cell a neurotrophic amount of a composition according to any one of claims 1-9.

11. A method for treating a patient suffering from neurological disorder comprising the step of administering to said patient a neurotrophic amount of a composition according to any one of claims 1-9.

12. A method according to claim 11 wherein said neurological disorder is selected from the group consisting of trigeminal neuralgia, glossopharyngeal neuralgia, Bell's Palsy, myasthenia gravis, muscular dystrophy, muscle injury, progressive muscular atrophy, progressive bulbar inherited muscular atrophy, herniated, ruptured or prolapsed invertebrae disk syndrome, cervical spondylosis, plexus disorders, thoracic outlet destruction syndromes, peripheral neuropathies caused by lead, dapsone, ticks, or porphyria, peripheral myelin disorders, Alzheimer's disease, Gullain-Barre syndrome, Parkinson's disease, Parkinsonian disorders, ALS, multiple sclerosis, central myelin disorders, stroke, ischemia associated with stroke, neural paropathy, neural degenerative diseases, motor neuron diseases, sciatic crush, neuropathy associated with diabetes, spinal cord trauma, facial nerve crush and other trauma, chemotherapy- or medication-induced neuropathies, and Huntington's disease.

13. A transgenic or chimeric non-human animal, comprising at least one isolated hFGF-8 polynucleotide, or complement thereof, encoding at least 90-100% of the contiguous amino acids of a protein selected from at least one of SEQ ID NOS:4, 5, 6, and 7.

14. The transgenic or chimeric non-human animal according to claim 13 wherein said hFGF-8 polynucleotide, or complement thereof, further comprises at least one mutation that results in at least one amino acid substitution, insertion, or deletion of said hFGF-8 polypeptide.

FIG. 1

Exon Representation of the FGF-8 Isoforms

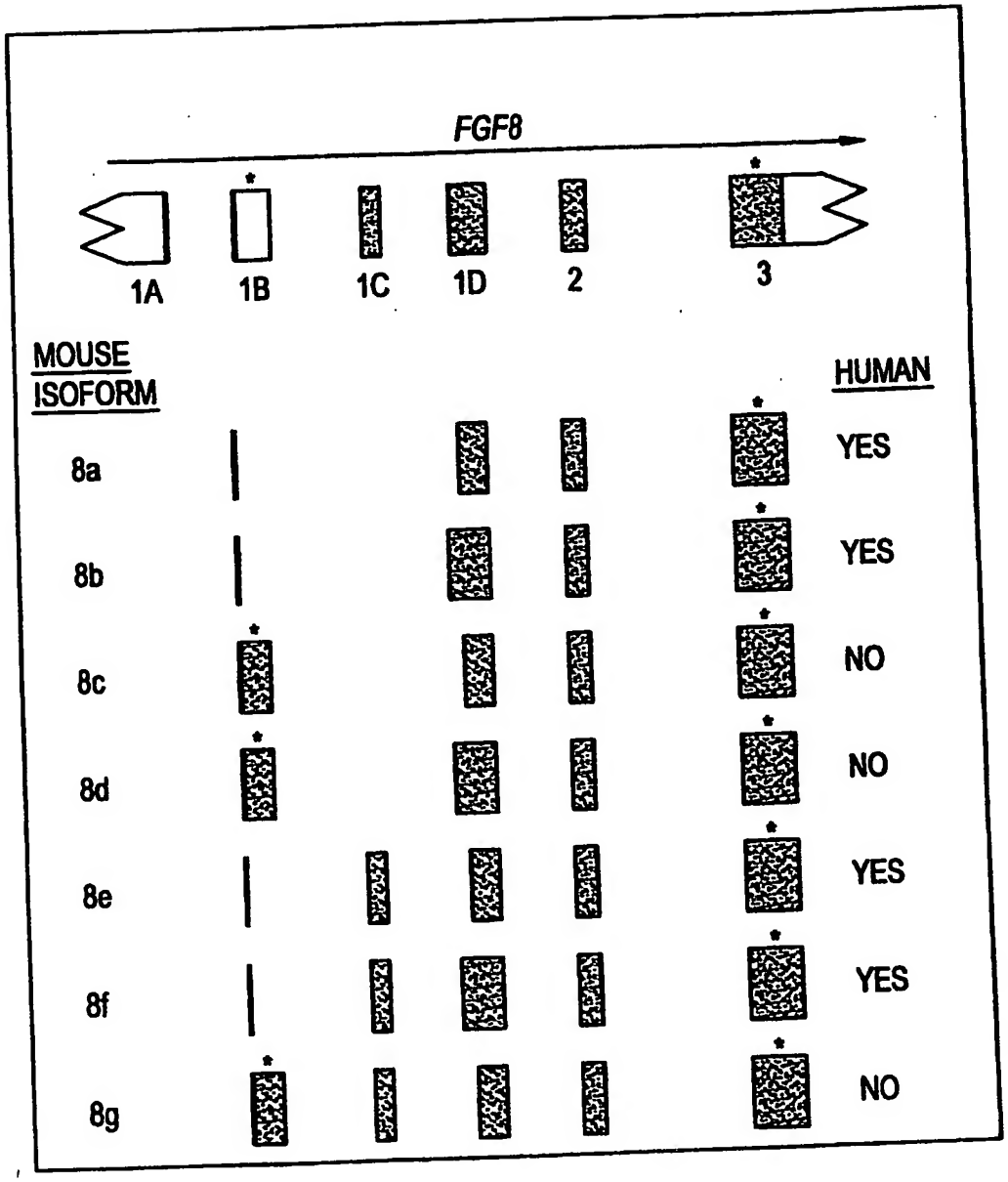
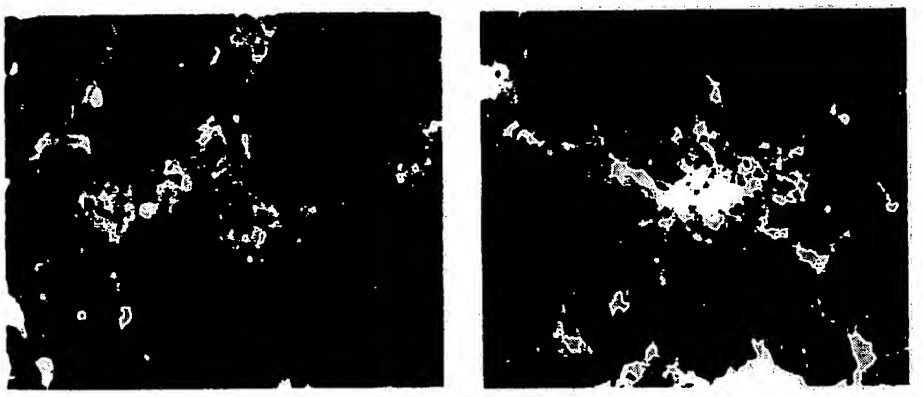
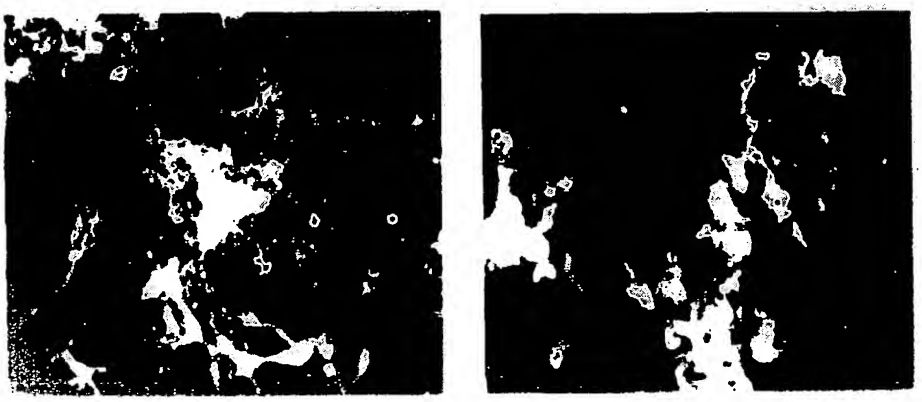


FIG. 2

Immunostaining of GAP-43 in SH-SY5Y

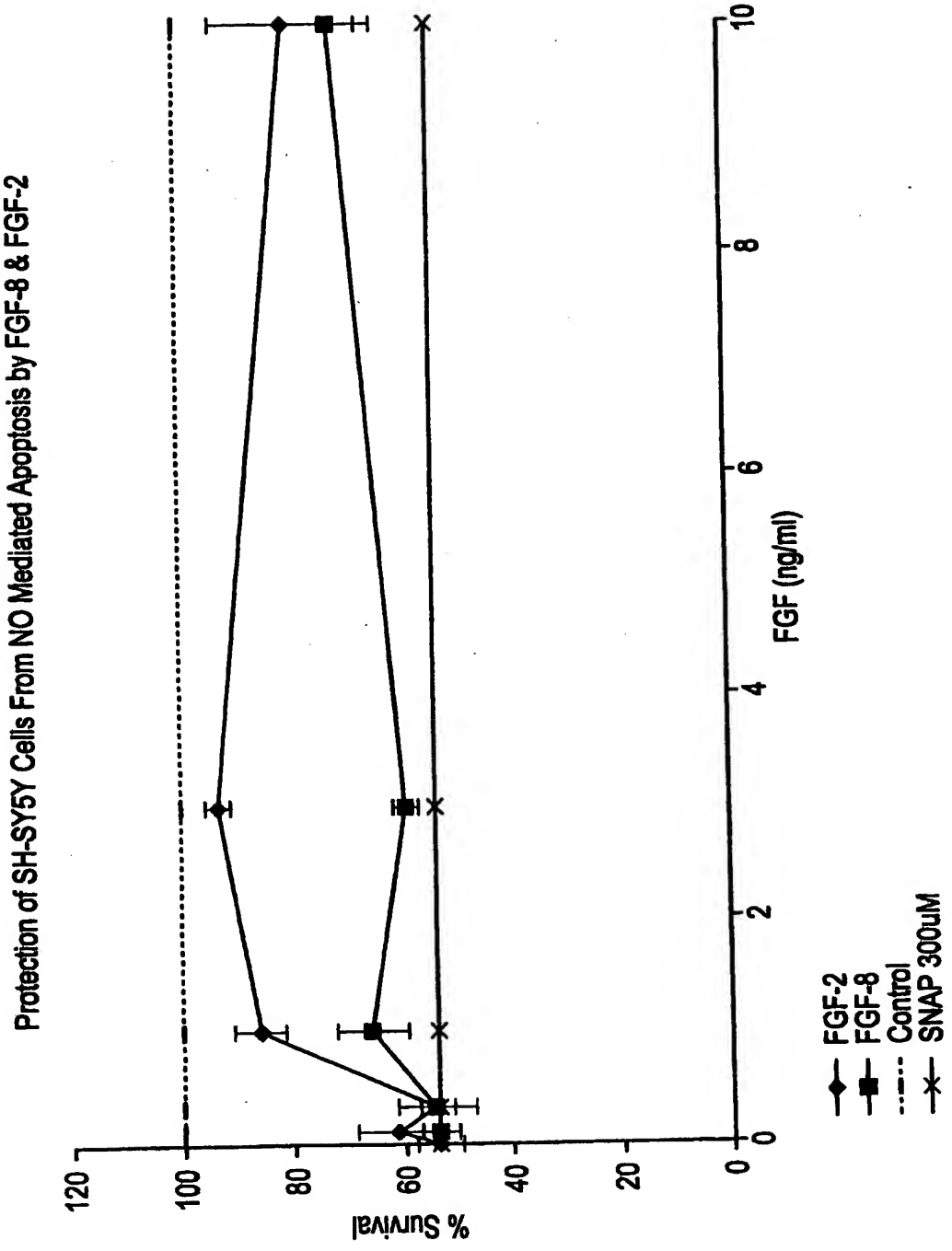


Control **FGF 2**



F8 **F8hc**

FIG. 3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FGF-8
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<212> PRT
<213> Homo sapiens

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Gly Arg Glu Leu Ala Ser Leu Phe Arg Ala Gly Arg Glu Pro Gln Gly
35 40 45

Val Ser Gln Gln His Val Arg Glu Gln Ser Leu Val Thr Asp Gln Leu
50 55 60

Ser Arg Arg Leu Ile Arg Thr Tyr Gln Leu Tyr Ser Arg Thr Ser Gly
65 70 75 80

Lys His Val Gln Val Leu Ala Asn Lys Arg Ile Asn Ala Met Ala Glu

85 90 95

Asp Gly Asp Pro Phe Ala Lys Leu Ile Val Glu Thr Asp Thr Phe Gly
100 105 110

Ser Arg Val Arg Val Arg Gly Ala Glu Thr Gly Leu Tyr Ile Cys Met
115 120 125

Asn Lys Lys Gly Lys Leu Ile Ala Lys Ser Asn Gly Lys Gly Lys Asp
130 135 140

Cys Val Phe Thr Glu Ile Val Leu Glu Asn Asn Tyr Thr Ala Leu Gln
145 150 155 160

Asn Ala Lys Tyr Glu Gly Trp Tyr Met Ala Phe Thr Arg Lys Gly Arg
165 170 175

Pro Arg Lys Gly Ser Lys Thr Arg Gln His Gln Arg Glu Val His Phe
180 185 190

Met Lys Arg Leu Pro Arg Gly His His Thr Thr Glu Gln Ser Leu Arg
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Phe Glu Phe Leu Asn Tyr Pro Pro Phe Thr Arg Ser Leu Arg Gly Ser
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Gln Arg Thr Trp Ala Pro Glu Pro Arg
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<213> Homo sapiens

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Val Leu Cys Leu Gln Ala Gln Glu Gly Pro Gly Arg Gly Pro Ala Leu
20 25 30

Gly Arg Glu Leu Ala Ser Leu Phe Arg Ala Gly Arg Glu Pro Gln Gly
35 40 45

Val Ser Gln Gln Val Thr Val Gln Ser Ser Pro Asn Phe Thr Gln His
50 55 60

Val Arg Glu Gln Ser Leu Val Thr Asp Gln Leu Ser Arg Arg Leu Ile
65 70 75 80

Arg Thr Tyr Gln Leu Tyr Ser Arg Thr Ser Gly Lys His Val Gln Val
85 90 95

Leu Ala Asn Lys Arg Ile Asn Ala Met Ala Glu Asp Gly Asp Pro Phe
100 105 110

Ala Lys Leu Ile Val Glu Thr Asp Thr Phe Gly Ser Arg Val Arg Val
115 120 125

Arg Gly Ala Glu Thr Gly Leu Tyr Ile Cys Met Asn Lys Lys Gly Lys
130 135 140

Leu Ile Ala Lys Ser Asn Gly Lys Gly Lys Asp Cys Val Phe Thr Glu
145 150 155 160

Ile Val Leu Glu Asn Asn Tyr Thr Ala Leu Gln Asn Ala Lys Tyr Glu
165 170 175

Gly Trp Tyr Met Ala Phe Thr Arg Lys Gly Arg Pro Arg Lys Gly Ser
180 185 190

Lys Thr Arg Gln His Gln Arg Glu Val His Phe Met Lys Arg Leu Pro
195 200 205

Arg Gly His His Thr Thr Glu Gln Ser Leu Arg Phe Glu Phe Leu Asn
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Tyr Pro Pro Phe Thr Arg Ser Leu Arg Gly Ser Gln Arg Thr Trp Ala
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Pro Glu Pro Arg

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<212> DNA
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cgggctggcc gggagcccca gggtgtctcc caacaggtaa ctgttcagtc ctcacctaat 180
tttacacagc atgtgaggga gcagagcctg gtgacggatc agctcagccg ccgcctcatc 240
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gtccacttca tgaagcggct gccccggggc caccacacca ccgagcagag cctgcgcttc 660
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ccggagcccc ga 732